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ACIDIC AND PHENOLIC FRACTIONS OF CRUCIFER ROOTS IN RELATION TO CLUBROOT [1]

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INTRODUCTION

One phase of a program of study of resistance in crucifers to the clubroot disease (*Plasmodiophora brassicae* Wor.) (*17*, *20*, *21*, *25*) [3] has been the biochemical investigation of the role of fungicidal constituents of the host plant. When Stahmann, Link, and Walker (*20*) found no evidence that either the nature or quantity of the mustard oils or the activity of myrosin, the mustard-oil glycosidase, was responsible for resistance, attention was directed toward the possible role of other types of compounds in the tissue which might contribute to resistance. The present paper presents the results of a study to determine the relation of the acidic and phenolic constituents of the root tissues to the resistance of crucifers to clubroot infection.

Acidic and phenolic compounds have received the attention of other workers in disease resistance. Cook and Taubenhaus (*2*, *3*) and others (*4*, *11*) [4] suggested that tannins or similar conjugated phenolic compounds were active in disease resistance. Dufrénoy (*6*) claimed that phenols were characteristic products of the cells of invaded tissues and that they might inhibit the spread of the pathogen. Kargapolova (*12*) reported that resistance in wheat to *Puccinia triticina* Eriks. was associated with special types of phenolic compounds in particular varieties. In studies of wheat rust (*Puccinia graminis* Pers.) by Newton and Anderson (*15*) and of sugarcane red rot (*Physalospora tucumanensis* Speg.) by Abbot (*1*), colorimetric estimates of phenols were reported to correlate with resistance. In only one case, however, has it been shown unequivocally that resistance is due to specific phenolic compounds. Walker, Link, and associates (*13*, *14*, *23*) showed that protocatechuic acid (3, 4-dihydroxybenzoic acid) and catechol (1, 2-dihydroxybenzene) were largely responsible for the resistance of pigmented onions to *Colletotrichum circinans* (Berk.) Vogl., the smudge organism. Other more strongly acidic compounds, such as carboxylic acids, have also been considered in resistance studies. Investigation of root rot (*Phymatotrichum omnivorum* (Shear) Dugg.) of cotton in Texas (*7*, *9*, *18*) has included the relation of acidic constituents of monocotyledonous plants to resistance as well as the effect of various acids and phenols on the causal organism itself.

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[2] The writers are indebted to Mark A. Stahmann for advice during this investigation.

[3] Numbers in parentheses refer to Literature Cited, p. 203.

[4] OFFORD, H. R. THE FUNCTION OF TANNIN IN HOST-PARASITE RELATIONSHIPS WITH SPECIAL REFERENCE TO RIBES AND CRONARTIUM RIBICOLA. U. S. Dept. Agr. Bur. Ent. and Plant Quar. E–518, 27 pp. 1940. [Processed.]

Preliminary work on various acidic fractions suggested two lines of approach. The first and most important was to determine whether there was a significant difference in fungicidal activity between extracts of resistant and susceptible turnip roots. The second was to determine whether the concentration of phenols, estimated colorimetrically, was correlated with resistance. These must necessarily have preceded any extensive efforts to isolate and identify the toxic materials. Both lines of approach were based on rather crude measurements, neither of which had any specific relation to the clubroot disease. Accordingly, to associate resistance clearly with such materials it was necessary, first, to demonstrate marked differences between resistant and susceptible turnip varieties in toxicity or in phenol content, and then to show that similar differences usually existed between resistant and susceptible varieties and species of the Cruciferae.

EXPERIMENTAL RESULTS

RESISTANT AND SUSCEPTIBLE HOSTS

Turnip (*Brassica rapa* L.) varieties previously tested by Walker (*21*) for resistance or susceptibility to clubroot were used as the source of plant extracts. In the beginning, the variety Purple Top Milan was selected as the resistant type inasmuch as it was shown to remain completely free from infection when exposed in a number of trials to the Wisconsin collection of the pathogen. This variety will be referred to hereafter in this paper as "R." Variety Shogoin was chosen as the susceptible host type because it consistently showed a high percentage of infected plants when inoculated with the pathogen. This variety will be referred to as "S." Later work was extended to other varieties of turnip and other crucifers, selected on the basis of their known resistance to the pathogen. Extra Early White Milan and Snowball turnip varieties were used as completely resistant varieties; Cowhorn was chosen as one which showed 25 to 40 percent susceptible individuals in the population (*21*).

Cabbage (*Brassica oleracea* var. *capitata* L.) was used as representative of very susceptible crucifers. A strain of rape kale (*B. napus* L.) found in numerous tests to be highly resistant was included. Two strains of black mustard (*B. nigra* L. Koch), the same as used by Pryor (*17*), were in one case highly resistant and in the other very susceptible to clubroot.

METHODS OF TOXICITY TESTING

Since toxicity to *Plasmodiophora brassicae* could not be conveniently or accurately measured, fungicidal activity was determined on *Colletotrichum circinans*. This organism had been used previously in testing the toxicity of phenols and mustard oils (*22*, *24*) and was shown to have sensitivity to the latter similar to that of *P. brassicae* (*10*). Two widely used types of assay were employed, the first based on the effect of toxicant on the rate of growth of the test organism, the second on the extent of germination of the spores.

The first type of assay used in the preliminary trials was similar to the technique of earlier work in this series (*22*, *24*) except that volumes were scaled down to 10 ml. per tube and observations were based on visual examination of growth rather than on mycelial weights. This technique had several disadvantages. It required 20 to 30 ml. of sample representing 200 to 300 gm. of fresh tissue and con-

siderable labor to set up, and it took several days to determine the results.

For these reasons the second type of test was developed, which was more convenient and economical in time and material (*19*). This technique was used routinely in all experiments reported here but was compared directly with the growth test on several typical extracts to make possible comparisons with earlier work. The following set of indices was used to express the results of the germination tests.

The inhibitory concentration, "IC," is that least concentration (grams dry weight per milliliter solution) of extract which caused complete (or nearly complete) inhibition of germination when the next lower concentration (in steps of one-half) allowed nearly complete germination. When the range from about 0 percent to about 100 percent germination was more than one concentration step, the IC was not considered fully reliable.

The toxicity level, "TL," which was equal to 1/IC, was thus a rough measure of the concentration of toxic substances in the fraction being tested. "TR" refers to the quotient of the TL of the resistant tissue extract divided by the TL of the susceptible tissue extract.

In experiments on methods of extraction and fractionation corresponding R and S fractions originally were compared as they occurred with the assumption that any differences in composition which caused differences in toxicity might be important in resistance. Consequently, no adjustment of pH, for example, was made. When it was later shown, however, that the concentration of strong acids altered toxicity of those fractions by shifting the pH (*19*), it was necessary to modify this assumption. Toxic substances effective in disease resistance in vivo would presumably be acting in the normal pH range of host cells, so it was decided to adjust all extracts to a uniform pH of 5.5 to 6.0. This cut down toxicity of most fractions but better approximated the conditions of host-parasite interaction in disease. The revised technique was used in the final extended analysis of tissues of a number of crucifers.

COLORIMETRIC METHODS FOR PHENOLS

It became evident early that several types of phenols, including the more labile polyphenols, occurred in turnip extracts but in relatively low concentration. It was necessary, therefore, to select analytical methods which were as general as possible without serious loss of specificity, were of maximum sensitivity, and were satisfactory for the labile polyphenols (ortho- and paradihydroxy-phenols). Folin's phosphomolybdic-phosphotungstic acid method and the diazo methods are the two most widely used colorimetric methods. However, they have been used largely with mono- and metadihydroxyphenols in animal tissues or in nonbiological materials, where purification is limited to precipitation of proteins or other nitrogenous-reducing compounds or to distillation of the phenols themselves. With plant material Newton and Anderson (*15*), employing the Folin method, used an ether-soluble, alkali-soluble fraction to avoid interfering compounds. They did not consider, however, the effect of alkaline extraction or the technique of color development on the labile polyphenols. The diazo methods are somewhat less general than the Folin method but because of their greater specificity can often be used with less purification of extracts.

Preliminary studies of the Folin and diazo colorimetric methods, which cannot be reported in detail, showed that neither was altogether satisfactory for the labile polyphenols. Alkaline extraction and small variations in the technique of color development caused marked changes in color values by both methods. The best results were obtained with the techniques described below, based on those of Folin and Ciocalteu (*8*) and Deichmann and Scott (*5*), when special care was exercised to maintain uniform conditions in the fractionation and color development.

Folin technique: Suitable volumes of an aqueous solution containing 1 to 5 mmM. (micromoles) of phenols were diluted to 20 milliliters. One milliliter of Folin-Ciocalteu reagent was added and the solution stirred. Five milliliters of 20 percent sodium carbonate was then quickly added and the solution again stirred. Finally, the solution was allowed to stand 30 minutes and the color read against a phenol standard in a Duboscq colorimeter. This standard containing 0.100 mmM. of phenol per milliliter was prepared daily from a stock solution of 1.00 mmM. of phenol per milliliter in 0.01 M sulfuric acid.

Diazo technique: The aqueous solution was neutralized with dilute sodium hydroxide to pH 6.5 to 7.0, or low enough to destroy the buffering capacity of the acids and permit the sodium acetate to raise it into this range. A suitable volume of solution to contain 0.25 to 2.0 mmM. of phenol was diluted to 20 ml., and 4 ml. of 25 percent sodium acetate containing 0.5 percent of gum acacia was added with stirring. Then 2 ml. of diazo reagent was added, the solution mixed and allowed to stand 1 minute, and 4 ml. of 20 percent sodium carbonate added with stirring. After 3 minutes the color was compared with a phenol standard as in the Folin technique. It was usually found necessary to make corrections for appreciable color in the controls. This color error was traced to the gum acacia but could not, however, be completely eliminated by several precipitations of the gum from alcohol-water mixtures. The error was compensated as indicated in the following formula:

$$Px = RxPs\left(\frac{1+Rc}{1-Rc}\right)$$

Where Px = true phenol content of sample;

$Rx = \frac{Px}{Ps+Pc}$ = colorimeter reading of sample against standard;

Ps = true phenol content of standard;

Pc = apparent phenol content of water control;

$Rc = \frac{Pc}{Ps+Pc}$ = colorimeter reading of water control against standard.

This correction often changed final values by 10 to 20 percent.

The phenol level, "PL," was expressed in micromoles per gram of fresh tissue. "PR" was the PL of resistant root extract divided by the PL of susceptible root extract.

The acidity level, "AL," was the titrable acidity in microequivalents per gram of fresh tissue. "AR" was AL of resistant root extract divided by the AL of susceptible root extract.

APPLICATION OF PHENOL METHODS TO FRESH TISSUE

Concurrently with the toxicant-fractionation experiments the phenol content of R and S fresh tissue was investigated by using the

two colorimetric methods and a fractionation scheme similar to Newton and Anderson's (*15*).

The first attempts were to measure only sap-soluble phenols, avoiding enzymatic or other hydrolytic action as far as possible. Fifty grams of the 2-mm. outer peeling of fleshy roots, consisting largely of periderm, phloem, and secondary xylem, were sliced, rapidly frozen with powdered dry ice, and the cold juice, expelled at 2,000 pounds per square inch, was immediately sucked into 25 percent trichloroacetic acid. The press cake was washed twice by soaking in the same solvent and repressing and the combined aqueous solution extracted with ether continuously in a liquid-liquid extractor. Comparison of apparent phenol content of press juice and ether extract by the diazo method showed that only 5 to 10 percent of the apparent phenols was extracted by ether so that further fractionation was necessary with both colorimetric methods. The ether concentrate was then extracted with 5 percent NaOH to give the "acidic-amphoteric fraction" which was acidified and again extracted with ether to give the "acidic fraction." Reproducibility and recovery of added phenols by this technique was only fair because of anomalous effects of alkaline extraction and occasional turbidity of final aqueous solutions. The PR ratio, the most reliable measure of relative phenol content of the two tissues, was generally greater than 1 but never significantly large (table 1).

TABLE 1.—*Phenol content of various fractions of fresh-tissue extracts from resistant (R) and susceptible (S) turnip root*

Extraction series Number	Source of extract	Ether-soluble fraction				Acidic-amphoteric fraction		Acidic fraction			
		Diazo method		Folin method		Diazo method		Diazo method		Folin method	
		PL [1]	PR [2]	PL [1]	PR [2]	PL [1]	PR [2]	PL [1]	PR [2]	PL [1]	PR [2]
3	R	0.13	1.6			0.11	1.6				
	S	.08				.07					
5	R	.12	1.4			.12	1.3	0.10	1.0		
	S	.085				.10		.10			
7	R	.15	1.7	0.28	1.3	.10	1.3	.04	.8	0.19	1.2
	S	.09		.22		.08		.05		.16	
8	R							.07	1.4	.23	1.0
	S							.05		.22	
8A [3]	R							.13	.8	.20	.9
	S							.16		.22	
9A [4]	R							.22	.9	.47	1.1
	S							.24		.41	

[1] PL=phenol level as micrograms of phenol per gram of fresh weight of tissue.
[2] PR=PL of R tissue divided by PL of S tissue.
[3] Extraction of press cake from series 8. [4] Autolyzed.

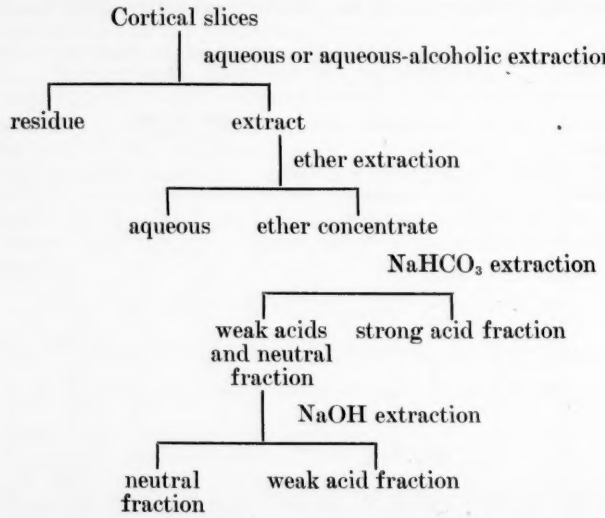
The lack of large differences between R and S in phenol content of press-juice fraction and previous experience with acid extractant suggested that greater differences might be found in unextracted or bound forms of phenols. Exhaustive alcohol extraction of the press cake showed that the major part of the tissue phenols was not in the press juice but could be removed by more thorough extraction. Fractionation and analysis of these extracts, however, showed no difference between R and S (table 1). Acid hydrolysis of press juice also failed

to give greater differences though there was a marked increase in phenol levels. Autolysis was also tried in a further effort to demonstrate differences in phenol content which might arise from hydrolytic or oxidative processes similar to those observed by Parker-Rhodes (*16*) with host extracts and rust uredospores. Tissue slices were frozen in dry ice and allowed to thaw and autolyze at room temperature for 48 hours before pressing and fractionating. Typical results in table 1 showed an increase in phenol concentrations as in the case of acid hydrolysis but no difference in PL's of R and S tissues.

These results of colorimetric analysis of phenols in fresh tissue indicated (1) no significant differences in press juice fractions and (2) increased yield without increased differences by alcohol extraction, acid hydrolysis, and autolysis.

EXTRACTION AND FRACTIONATION

An extensive series of experiments which will not be reported in detail was carried out to determine the effect of various methods of extraction and fractionation on the relative fungicidal activities of R and S tissues. Aqueous and 30 percent alcoholic extracts of dried cortical tissues, both neutral and acid, from 1938 and 1939 crops were fractionated by the following scheme.



The results in brief were (1) PR's were consistently greater than 1 only in the strong acid fractions, but the differences were never in proportion to the differences in disease resistance; (2) acid extraction under mild conditions gave higher PL's and higher PR's than neutral conditions, while more drastic acid conditions diminished the PR's; (3) alcoholic extraction cut down the interference of pectic materials in the ether extraction step but gave no higher yield of fungicidal materials.

The technique of extraction and fractionation finally adopted for extended analysis of crucifer tissue and designated as the PT method

was as follows. Sliced, fresh cortical peelings in 500-gm. lots were refluxed in quart mason jars for 30 minutes in an aqueous solution of 0.5 percent sulfuric acid and 45 percent alcohol (including tissue water). After 8 months' storage at room temperature each lot was filtered, homogenized in a Waring blendor with fresh solvent, and the pulp squeezed in a hand screw press. The combined alcoholic extracts were concentrated *in vacuo* at 25° to 30° C. to about 100 ml., diluted with water, and reconcentrated twice to remove all alcohol. The resulting aqueous suspension was cooled to 6° C. overnight and filtered through a mat of Filter Cel to remove as much of the gummy, water-insoluble material as possible. The filtrates were then diluted to about 800 ml. and extracted with ether for 36 hours in a continuous liquid-liquid extractor. The ether extracts were made up to 200 ml. and shaken six times with 100 ml. of 5 percent sodium bicarbonate for 4 minutes, each extract being run quickly into excess dilute sulfuric acid. These acidified solutions were again extracted continuously with ether for 24 hours and the resulting "strong acid fraction" dried over sulfuric acid, concentrated to 50 ml., and stored at 6° C.

Limited application of the PT method to R and S tissue of the 1940 crop was made and germination tests, phenol analyes, and acidity titrations were run on aqueous solutions made by suspending aliquots of the ether concentrates in water and evaporating the ether. Titration curves of various lots were found to be similar, so titrable acidity was measured as the micro equivalents of acid per gram of fresh tissue titrated to an equivalence point of about pH 8. In several cases large differences in fungicidal activity, in phenol content, and in titrable acidity were found between R and S tissue (table 2).

TABLE 2.—*Typical results of strong acid fractions from extraction and fractionation of R and S tissues by the PT method*

Series No.	Source of extract	Fungicidal activity		Phenol content				Titrable acidity	
				Diazo method		Folin method			
		TL[1]	*TR*[1]	*PL*[1]	*PR*[1]	*PL*[1]	*PR*[1]	*AL*[1]	*AR*[1]
PT2	R	16–32	8–16	1.3	3.8	1.1	3.2	16.3	22
	S	2		.35		.36		.74	
PT4	R	32	8+	.13	5	.48	5	11.5	30
	S	2–4		.026		.10		.37	

[1] For explanation of symbols, see Methods of Toxicity Testing, p. 194, and Colorimetric Methods for Phenols, p. 195.

In assaying these particular strong-acid fractions it was also observed for the first time that the fungicidal activity varied markedly with the pH of the test solution. Toxicity of these fractions increased with increasing hydrogen-ion concentration from pH 6 to 3, its behavior in this respect being similar to that of carboxylic acids (*19*). In fractionation these acids had been separated from the natural buffers of the tissue, and in the unbuffered test solutions they were acting at pH values considerably below the physiological range of the host tissues. Thus strong acids by lowering the pH exerted a misleading indirect effect on the fungicidal activity of any compounds, such as carboxylic acids and phenols, whose toxicity varied with hydrogen-ion concentration. For this reason all subsequent toxicity assays were made in buffered solutions.

EXTENDED ANALYSIS OF CRUCIFER EXTRACTS BY THE PT METHOD

An extensive test of the methods of the previous section was carried out on a new lot of tissue grown in the summer of 1941 along with some of the 1940 lot. Cabbage and rape, kale, several varieties of turnip, including the R and S varieties, and a resistant and a susceptible strain of mustard were preserved by the PT method, both in neutral alcohol and acid alcohol containing 0.5 percent sulfuric acid. Several samples of each variety and each treatment were fractionated and strong-acid and weak-acid fractions prepared.

Phenol analyses were run two to four times on each of two aliquots of the ether concentrates. The analyses were carried out in large numbers so that many samples could be compared directly with each other without depending upon the less accurate comparison of extracts with pure phenol standards. Different runs were, however, all calculated to a phenol basis. This method of comparing different aliquots of the same ether concentrate or of the same aliquot analyzed at different times showed variations of less than 10 percent, which was considered definitely less than the errors due to fractionation of and variation in tissue. Toxicity measurements were carried out by the revised germination technique eliminating the pH effect and were expressed as means of two to four separate tests.

TABLE 3.—*Summary of analyses by the PT method and toxicity tests of several crucifers*

Plant species and variety	Resistant or susceptible	Year sample was grown	Type of fraction	Experiment No.	Phenol content (PL) [1]		Titrable acidity (AL) [1]	Toxicity (TL) [1]
					Diazo method	Folin method		
Turnip (var. Purple Top Milan).	Resistant	1940	Acid-alcohol; strong acid.	7A	0.23	0.69	20.0	2-4
		1940	do	7B	.21	.68	21.0	4—
		1940	do	9A	.059	.020	.32	4
		1940	do	9B	.033	.11	.82	1-2
		1940	do	9C	.21	.60	16.6	2
		1941	do	10B	.23	.75	19.5	2-4
		1941	Neutral-alcohol; strong acid.	11A	.088	.24	.65	
		1941	do	11B	.14	.32	.65	2—
		1941	Acid-alcohol; weak acid.	10A	.011	.04		
		1941	do	7A	.014	.054		
Turnip (var. Shogoin)	Susceptible	1941	Acid-alcohol; strong acid.	4bA	.17	.64	13.0	
		1940	do	4dA	.19	.20	.32	2-4
		1940	do	4dB	.19	.55	14.6	2
		1941	do	13A	.066	.23	6.5	
		1941	do	15A	.10	.32	7.8	2
		1941	Neutral-alcohol; strong acid.	10A	.079	.31		2
		1941	do	10B	.11	.29	1.3	2+
		1941	do	12A	.019	.042	.26	
		1941	do	4dB	.023	.082		
Turnip (var. Cowhorn)	Partially resistant.	1941	Acid-alcohol strong acid.	25A	.19	.64	16.1	2
		1941	do	25B	.32	1.09	9.6	4—
		1941	Neutral-alcohol; strong acid.	24A	.091	.34	.91	
		1941	do	24B	.080	.22	.97	
		1941	Acid-alcohol; weak acid.	25A	.017	.071		
Turnip (var. Extra Early White Milan).	Resistant	1941	Acid-alcohol; strong acid.	21B	.27	.80	18.0	4
		1941	do	21C	.31	.98	19.5	4
		1941	Neutral-alcohol; strong acid.	20A	.17	.43	.53	
		1941	do	20C	.09	.25		

See footnotes at end of table.

TABLE 3.—*Summary of analyses by the PT method and toxicity tests of several crucifers*—Continued

Plant species and variety	Resistant or susceptible	Year sample was grown	Type of fraction	Experiment No.	Phenol content (PL) [1]		Titrable acidity (AL)[1]	Toxicity (TL)[1]
					Diazo method	Folin method		
Turnip (var. Snowball).	Resistant	1941	Acid-alcohol; strong acid.	23A	0.35	1.16	15.6	2-4
		1941	do	23B	.29	1.05	14.8	2-4
		1941	do	23C	.32	.84	14.6	-------
		1941	Neutral-alcohol; strong acid.	22A	.073	.18	.97	-------
		1941	do	22B	.084	.25	.97	-------
		1941	Acid-alcohol; weak acid.	22B	.017	.041	-------	-------
Rape kale	Resistant	1941	Acid-alcohol; strong acid.	31A	.25	.93	13.9	4—
		1941	do	31B	.27	1.04	11.0	-------
Cabbage	Susceptible	1941	Acid-alcohol; strong acid.	33A	.19	.56	5.06	2
		1941	do	33B	.16	.49	-------	-------
Black mustard	Resistant	1941	Acid-alcohol; strong acid.	-------	.33	1.13	14.5	1-2
	Susceptible	1941	do	-------	.25	.91	13.7	2—

[1] For explanation of symbols see Methods of Toxicity Testing, p. 194, Colorimetric Methods For Phenols, p. 195.

The results summarized in table 3 show no significant correlation between IC's and resistance—nearly all assays lay within the narrow range of 2 to 4. Buffering the test solutions to eliminate the pH effect had markedly reduced the TL's as expected. Titrable acidity, likewise, showed no clear correlation with resistance though there was fair agreement among replicates, with a few exceptions. These exceptions were acid-alcohol, strong-acid fractions whose AL's dropped into the range of the neutral-alcohol fractions. Whether this was due to failure of the 0.5 percent sulfuric acid in these cases to exert its usual effect on strong-acid extraction or to tissue variation could not be determined. The large differences in phenol content between resistant and susceptible host tissues of the 1940 trials were also not confirmed and considerable variation among replicates was apparent. A few replicates consistently fell below others in the treatment, somewhat as in the case of titrable acidity, showing, in fact, a fair correlation between phenol and strong-acid content. It must be assumed that these represent either tissue variation or some unknown discrepancy in the fractionation. Disregarding such samples, however, would not alter the over-all interpretation of the results. Neutral extraction again gave variable but generally lower phenol content than acid extraction. Representative weak-acid fractions showed usually less than one-tenth the phenol content of corresponding strong-acid fractions. The major phenolic compounds of these crucifer extracts were apparently strong enough acids to extract into the sodium-bicarbonate-soluble acid fraction.

DISCUSSION

Previous work in this laboratory had suggested that other fungicidal constituents of crucifers than the mustard oils might be involved in resistance to clubroot and certain evidence pointed to acidic or

phenolic fractions. The first objective of the present work, accordingly, was to determine whether there was a significant correlation between fungicidal activity of extracts or particular fractions and the resistance of crucifers in the field. An attempt was made at the same time to determine whether phenolic compounds, specifically, were involved by colorimetric estimation in several types of crucifer extracts.

Study of the effect of extraction and fractionation methods on toxicity confirmed preliminary evidence that the ether-soluble, strong-acid fraction showed the most consistent differences in toxicity between extracts from resistant and susceptible turnips. Acid extraction or hydrolysis tended to increase toxicity levels of extracts of both types of tissue and under certain conditions seemed to increase differences between them. In fact, one method involving acid extractants (PT method) with turnip tissue of the 1940 crop showed very significant differences in fungicidal activity, phenol content, and titrable acidity. More important, however, these experiments revealed that the titrable acidity of acid fractions varied considerably in different tissue samples and caused marked variation in the pH of extracts used in toxicity testing. Study of the influence of pH on the toxicity of turnip extracts (*19*), moreover, showed that the greater part of the toxicity of unbuffered extracts was probably due to this increased fungicidal activity of strong acids at low pH values.

More extensive analyses of several crucifers from the 1941 crop showed no significant correlation between toxicity and resistance. It seemed probable that the several cases of large differences in toxicity of extracts of resistant and susceptible turnips in preliminary trials were due mainly to differences in acid content. With the pH effect of acids on toxicity eliminated it is doubtful whether such correlation would ever have appeared.

Application of Newton and Anderson's method of extraction of phenols by using both the Folin and diazo colorimetric methods on fresh turnip tissues showed a possible correlation of sap-soluble phenol content with resistance. It was found, however, that the major part of the extractable phenols was freed only by acid hydrolysis or autolysis. These methods, however, still produced no clean-cut correlation of phenol content and resistance. The phenol levels found were close to those from autolyzed fresh tissue and the ratio of levels by the Folin and diazo techniques was similar to those in press-juice fractions. It should also be pointed out that the average concentration of phenols found in crucifer roots was somewhat less than that reported by Newton and Anderson (*15*) in wheat seedlings and probably less than one-tenth the amounts shown by Link and associates (*13*, *14*) to be present in the outer scales of onions and to be effective in resistance to smudge. The correlation of phenol content in crucifer roots with clubroot resistance is not as strong as in the case of onion smudge (*13*, *14*) and the relatively low concentrations present might argue against any relation with disease resistance. As pointed out in another paper (*19*), however, small differences in hydrogen-ion concentration of resistant and susceptible tissue may cause relatively large differences in the fungicidal effectiveness of certain cellular constituents. Although present evidence fails to demonstrate that resistance to clubroot in crucifers is due in any large part to readily extractable phenolic or acidic toxic constituents of the root tissues, the possibility

still remains that fungicidal compounds are produced during invasion of the pathogen in response to special stimuli or are caused by derangement of normal metabolism. The methods employed in the present investigation would hardly have detected mechanisms of this kind. Adequate study of such complex changes in invaded tissues with the possible production of fungicidal constituents will depend upon better understanding of the metabolism of normal tissues.

SUMMARY

The suspected significance of phenolic or acidic constituents of root tissues in resistance to clubroot of crucifers was investigated by phenol and acid analysis and toxicant fractionation.

Methods of phenol determination in plant tissue were studied, and modified Folin and diazo techniques were applied to the estimation of phenol content of fresh tissues. No clean-cut correlation was found between resistance and sap-soluble phenols of resistant (Purple Top Milan) and susceptible (Shogoin) turnip varieties. Exhaustive alcohol extraction, acid hydrolysis, and autolysis caused increased yields of phenols in both varieties but no significant differences.

Study of extraction and fractionation techniques confirmed the presence of toxic materials in the ether-soluble, sodium-bicarbonate-soluble fraction. These fractions were assayed for fungicidal activity by their effect on percentage germination or growth of *Colletotrichum circinans*. The most promising technique, the PT method, in limited application to the two turnip varieties of the 1940 crop, showed highly significant differences in phenol content, titrable acidity, and toxicity which correlated with resistance. It was discovered, however, that at least part of the toxicity of these fractions was due to the effect of strong acids on the pH of test solutions. Extensive application of the PT method to representative crucifers of varying field resistance from the 1941 crop and elimination of the pH effect in toxicity testing failed to show any clear correlation between phenol content, acidity, and fungicidal activity and resistance.

The results did not show that resistance to clubroot of crucifers was due in any large part to the presence of preformed extractable phenolic or acidic fungicidal constituents in the cortical tissues of the fleshy roots.

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STUDIES ON THE INHERITANCE OF HARD SEEDS IN BEANS [1]

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INTRODUCTION

The presence of hard seeds in many legumes is a serious problem to farmers, to seed dealers, to canners, and to consumers. In most States regulations for enforcement of seed laws specify tolerance of hard seed in various legumes. To reduce the number of hard seeds in small-seeded legumes, various methods of seed treatment, such as scarification or treatment with acids or hot water, have been applied. However, these practices are time consuming, expensive, and tend to mutilate the seeds. Bean canners have demanded that seedsmen eliminate hard seeds, and often require that the seed purchased be relatively low in moisture. To meet this latter requirement dealers dry seeds artificially, which tends to increase the number of hard seeds.

In previous studies [2] of the effect of heredity and environment on the production of hard seeds in beans (*Phaseolus vulgaris* L.), the seeds of 36 selections of common white beans were dried over calcium chloride to 10 different moisture contents, ranging from 14.11 to 5.59 percent. There were no hard seeds in the control which contained 15.14 percent of moisture, but lots of seed with 14.11 percent of moisture contained 1 percent of hard seeds. With each reduction in seed-moisture content the percentage of hard seeds increased and the seeds remained hard for a longer time. When moisture content was reduced to 5.59 percent nearly 90 percent of the seeds were hard.

Individual selections differed in regard to hard-seed production. Some selections developed a greater percentage of hard seeds in seed of the same moisture content; and in some selections hard seeds softened readily when tested for germination while in others they remained hard for a long time. These differences among individual selections in regard to hard-seed production were found to be hereditary.

In the present report the softening of hard seeds in the F_1 and F_2 generations of certain crosses is described.

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[1] Received for publication May 10, 1945. Paper No. 139, Journal Series, Georgia Agricultural Experiment Station, Experiment, Ga.

[2] LEBEDEFF, G. A. HEREDITY AND ENVIRONMENT IN THE PRODUCTION OF HARD SEEDS IN COMMON BEANS (PHASEOLUS VULGARIS). Puerto Rico (Rio Piedras) Univ. Agr. Expt. Sta. Res. Bul. 4, 27 pp., illus. 1943.

MATERIAL AND METHODS

Out of 36 selections studied previously, 6 selections, Nos. 12, 368, 475, 1083, 1099, and 1130, were chosen for further study. A detailed description of hard-seed production in these selections can be found in the publication referred to above.

Many crosses were made between these selections. The F_1 plants, together with several plants of each parental selection, were grown in pots outdoors in partial shade during the spring of 1943 in Puerto Rico. The seeds were harvested about June 1 and stored in the laboratory. On August 15 these seeds, as well as extra seeds of the original crosses, were taken to Georgia where further studies were made under different environmental conditions and with modified methods.

Arrangements were made at Experiment, Ga., to grow the original 6 selections and the F_1 and F_2 plants from crosses between these selections at the same time and under similar conditions. Two greenhouses were available for this purpose. The seeds from crosses were planted on November 15 to grow the F_1 plants. At the same time the seeds from 6 selections and those from the F_1 plants were germinated in the laboatory (prior to germination the moisture content of this material was reduced to about 9 percent over $CaCl_2$),[3] and the seedlings were transplanted to pots in the greenhouse. A total of 750 F_2 plants, over 100 F_1 plants, and over 100 plants of the 6 selections were grown in the greenhouses.

The seed from each plant was harvested separately during February 1944. All the threshing was done by hand, and the seeds from individual plants were put into individual cheesecloth bags and kept in the laboratory for about 3 months. Disregarding about 100 lots with less than 25 seeds which were discarded, the average number of seeds per plant was for the selections, 49.7; for the F_1 plants, 53.3; and for the F_2 plants, 45.2. Each curve in figures 1 to 5, therefore, is based on an average of about 50 seeds.

In order to induce hard-seed development in these 850 lots of seed, they were placed in an electric dehydrator of a cabinet type designed for home drying of fruit and vegetables.[4] The seeds, still in bags, were placed on seven trays inserted one above another in the drier. The trays were interchanged daily, and at the same time the bags were turned over to insure uniform drying. The temperature of the air in the drier remained approximately 45° C.

Several bags of seed were removed from the drier after 1, 2, and 3 days of drying and their moisture content was determined. After 5 days the drying was discontinued, and the seed lots, with the exception of 20 bags, were placed in an electric germinator. The moisture content of these 20 samples was determined, and this was taken to represent the approximate moisture content of the rest of this material. For determination of moisture content these 20 seed lots were crushed in a small meat chopper and then kept for 48 hours in an electric oven at 110° C. After the samples had been removed from the oven they were cooled in a desiccator and then weighed. The mean moisture content of the 20 samples was 6.61 ± 0.121 (S. D. $= 0.54 \pm 0.085$).

[3] Records were kept of hard-seed production in these seeds, but they are not reported here because more extensive data were obtained later.

[4] SHUEY, G. A. DEHYDRATION OF FRUITS AND VEGETABLES IN THE HOME. Tenn. Agr. Expt. Sta. Bul. 183, 21 pp., illus. 1943.

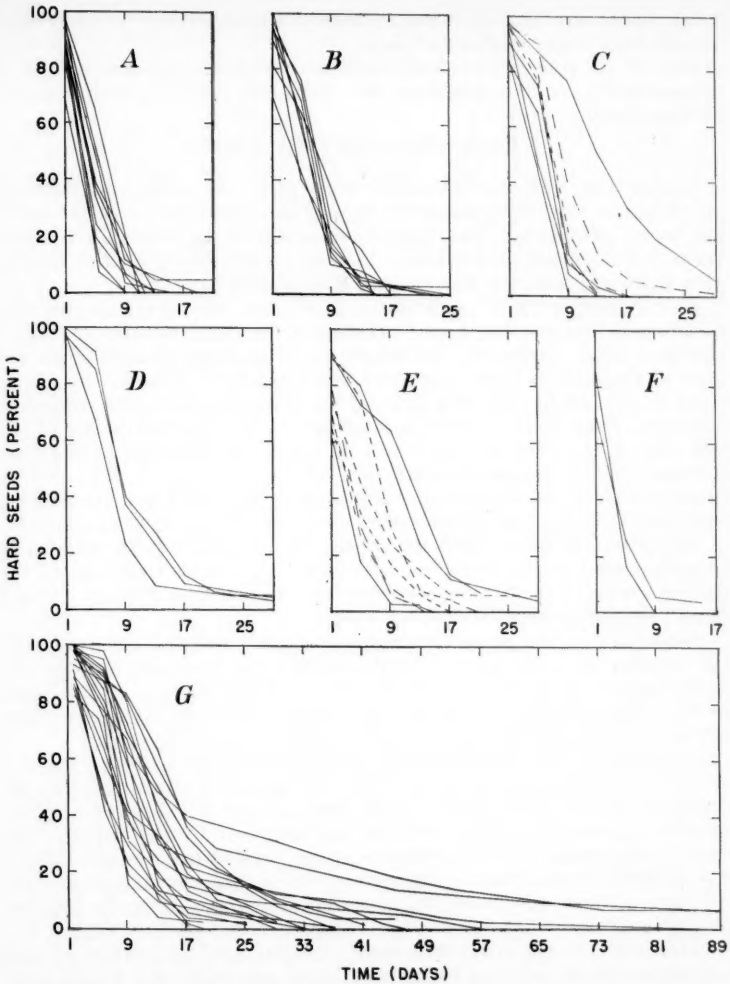

FIGURE 1.—Percentage of hard seeds on successive days of tests. *A*, selection 1099; *B*, selection 368; *C*, F_1 from 1083×1099 (solid lines) and reciprocal crosses (broken lines); *D*, F_1 from 475×1099 crosses; *E*, F_1 from 1083×368 (solid lines) and reciprocal crosses (broken lines); *F*, F_1 from 1099×368 crosses; *G*, F_2 from 475×1099 crosses. The F_1 and F_2 seeds from various crosses in figures 1 to 5 refer to the seed produced by the F_1 and F_2 plants respectively.

All germination tests were made in a Minnesota electrically heated germinator, model No. 3, in which the air temperature was approximately 28° C. The remaining 800 lots of seed were removed from the cheesecloth bags and placed in the germinator on the wire mesh trays which were covered with two thicknesses of cotton flannel cloth. At the beginning of the test, counts of hard and swollen seeds were made

every other day, and the swollen seeds were removed. After 35 days counts were made every 4 to 8 days.

Out of six selections which were intercrossed, only the results of crosses involving five selections, Nos. 1099, 368, 1083, 475, and 1130, will be described.

HARD SEEDS IN PURE LINES

Under the new environment and with the different methods employed in this experiment for drying and germinating of the seeds, hard-seed production was somewhat modified in several selections from that reported previously.[5] It is suspected that the quick drying at a high temperature was mainly responsible far this.

The 2 "soft-shelled" selections, 1099 and 368, were practically unchanged and the hard-seed production in them closely resembled that previously reported. Although the proportion of hard seeds was high in the seed of these selections, the hard seeds softened in a very short time. More than 20 plants of the 1099 selection were available, and seeds from most of them became soft by the thirteenth day of the test (fig. 1, *A*). In the 20 plants of selection 368 most of the seed became soft by the seventeenth day of the test (fig. 1, *B*). While there was a certain amount of variability in the hard-seed production from individual plants of both selections, the differences were slight.

All three "hard-shelled" selections, namely, 475, 1083, and 1130, became considerably harder in this experiment than they had in the earlier studies, and the time required for the softening of hard seeds of these selections was considerably longer.

Selection 475 was most drastically changed, and the usual drop in the number of hard seeds expected during the first few days of the germination test did not occur at all. Instead, the number of hard seeds in this selection decreased very slowly, and even after more than 200 days a large percentage of seeds remained hard (fig. 2, *B*).

In selection 1083 the softening of seeds was prolonged also but not to the same extent as in selection 475. In the earlier experiments conducted in Puerto Rico most of the seed of this selection at approximately the same moisture content had softened by the twenty-fifth day of the germination test, whereas, in the present experiment, softening of seeds of some plants of this selection was not complete even after 80 days. There was, however, considerable variation in the production of hard seeds in the individual plants in selection 1083 (fig. 4, *D*).

Selection 1130 appeared to be least changed, and the graphs of seed softening almost parallel those previously reported (fig. 3, *A*).

INHERITANCE OF HARD-SEED SOFTENING IN CROSSES

CROSSES INVOLVING SELECTIONS 1099 AND 368

The rate of seed softening in selections 1099 and 368 was essentially the same. The proportion of hard seeds in these selections usually was large, yet the hard seeds softened very quickly. Despite their similar behavior, these two selections were found to be different genetically in regard to hard-seed production. In crosses with 475, selection 1099 was found to be nearly completely dominant, whereas selection 368 was nearly recessive.

[5] See footnote 2, p. 205

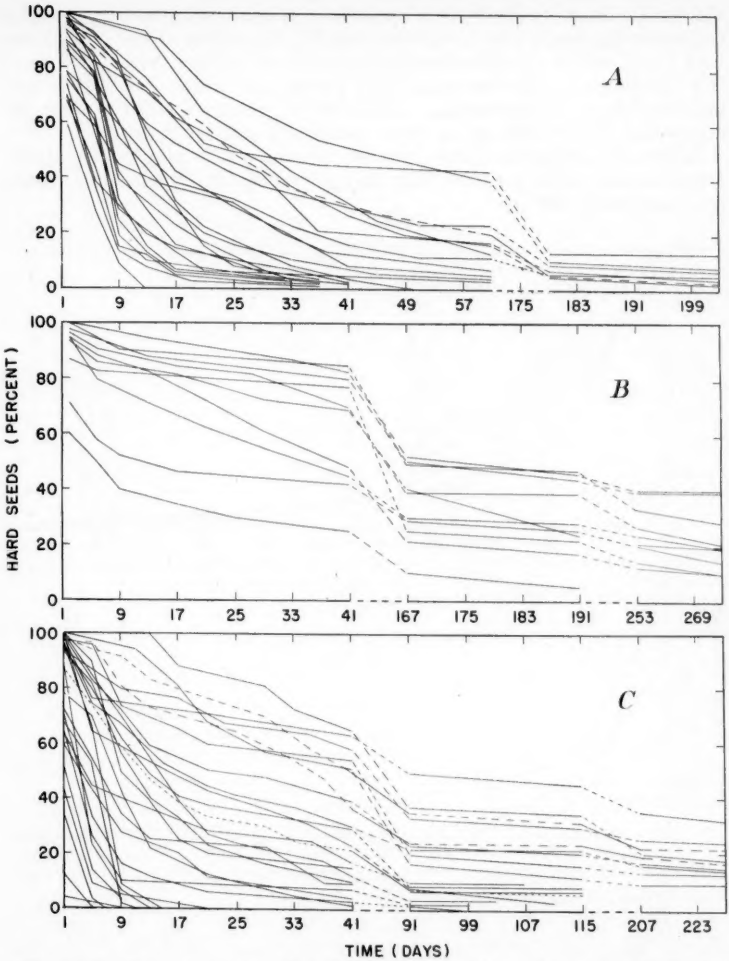

FIGURE 2.—Percentage of hard seeds on successive days of tests. *A*, F_1 (broken line) and F_2 (solid lines) from 368×475 crosses; *B*, selection 475; *C*, F_1 (broken lines) and F_2 (solid lines) from 1083×1130 crosses.

In the F_1 from the 475×1099 cross only 3 plants were available (fig. 1, *D*), and the seeds from these plants softened almost as rapidly as those of the 1099 parent. Twenty-one F_2 plants were available from the 475×1099 cross (fig. 1, *G*). In this population possibly 1 or 2 plants could be classified as producing hard seeds similar to those of the 1099 parent. In the rest of the plants the seeds softened considerably more slowly than those of the 1099 parent, but they were not nearly as slow in softening as those of the 475 parental selection.

In the 1 F_1 plant available from the 368×475 cross the seed softening was nearly as slow as that of seeds of the 475 parent (fig. 2, *A* and *B*).

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Of the 22 plants in the F_2 from the 368×475 cross, 1 could be classified as producing seeds which softened readily like those of the 368 parent, and 1 or 2 which produced seeds that softened at the very slow rate of the 475 parent. In the remaining plants the seed softening ranged between these two extremes. Eighteen F_2 plants available from the reciprocal (475×368) gross gave essentially similar results.

Although selections 1099 and 368 proved to be genetically unlike when crossed with selection 475, they gave practically the same results in crosses with 1083.

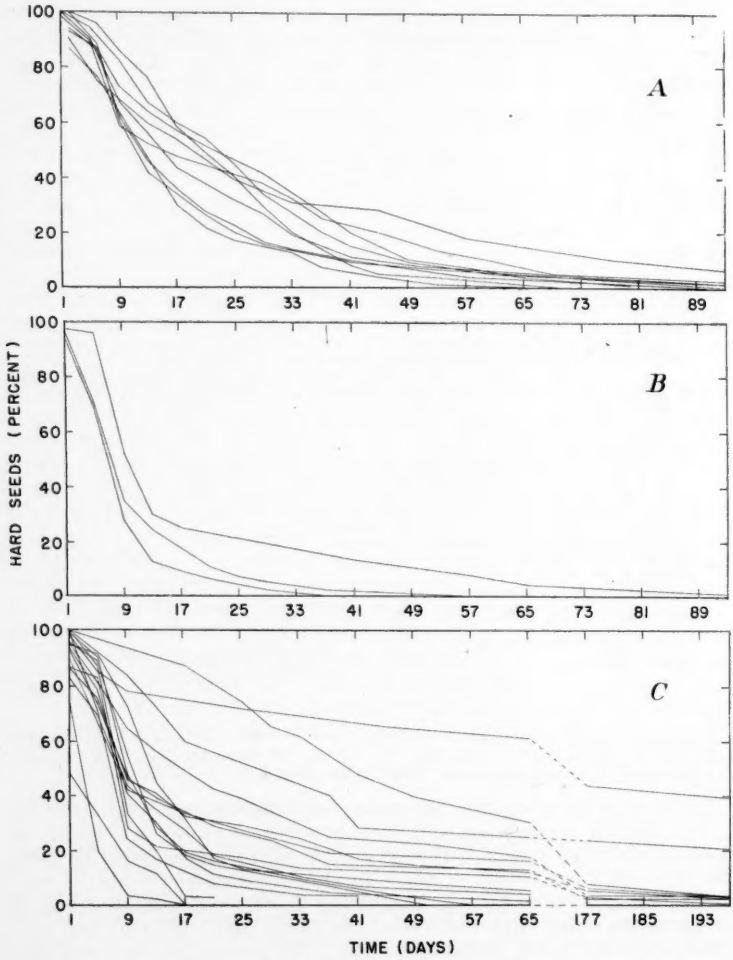

FIGURE 3.—Percentage of hard seeds on successive days of tests. *A*, selection 1130; *B*, F_1 from 368×1130 crosses; *C*, F_2 from 368×1130 crosses.

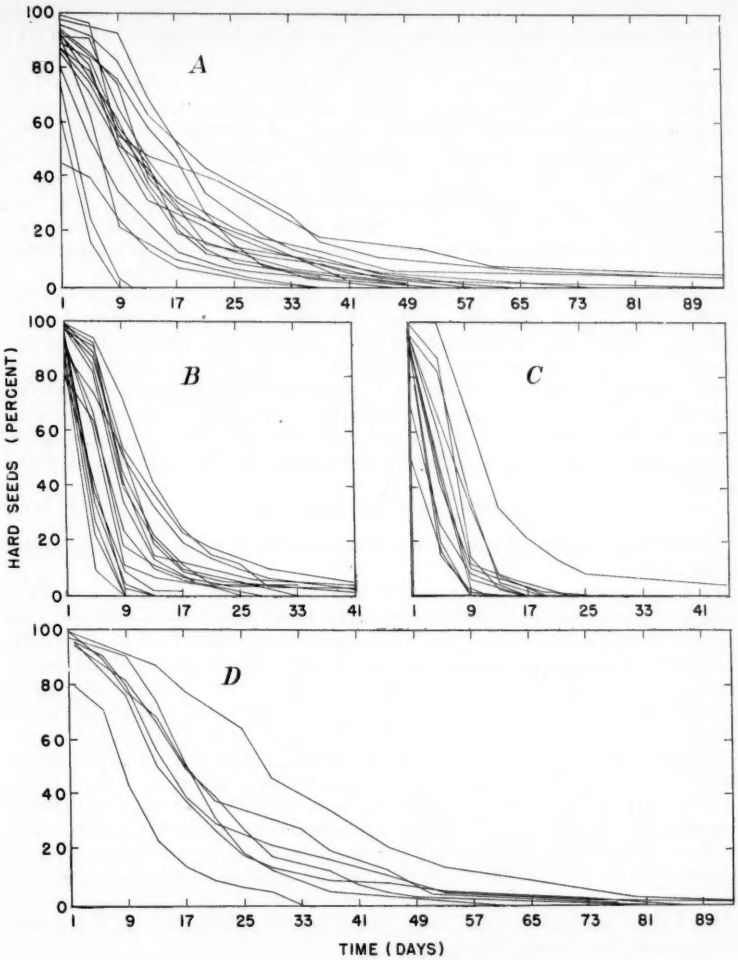

FIGURE 4.—Percentage of hard seeds on successive days of tests. *A*, F_2 from 1083×1099 crosses; *B*, F_2 from 1099×1083 crosses; *C*, F_2 from 1099×368 crosses; *D*, selection 1083.

There were 8 F_1 plants available from the 1099×1083 and reciprocal crosses (fig. 1, *C*). In 7 plants seed softening was very rapid, similar to or approaching that of the 1099 parent, while in 1 plant it was slower and approached that of the 1083 parent. In 17 F_2 plants from the 1083×1099 cross, seed softening varied from the very fast, as in 1099 to that of the slower softening 1083 parent, with the majority of plants showing seed softening of variously intermediate rates (fig. 4, *A*). In the F_2 from the reciprocal cross (1099×1083) essentially sim-

ilar segregation occurred but with less dispersion and with the graphs of seed softening inclining toward that of the 1099 parent (fig. 4, *B*).

There were 8 F_1 plants available from the 368×1083 and reciprocal crosses (fig. 1, *E*), and 22 F_2 plants from the 1083×368 crosses (fig. 5, *A*). The seed softening in both F_1 and F_2 plants from these crosses was very similar to that found in crosses of selections 1099 and 1083.

The cross between the two "soft-shelled" selections 1099 and 368 was not well represented. In the F_1 from the 1099×368 cross, seeds of only 2 plants were available, and seed softening in them was like that of the 1099 parent (fig. 1, *F*). Seeds of 13 F_2 plants were available, and seed softening varied between that of the 2 fast softening parental selections or slightly exceeded that of the 368 parent (fig. 4, *C*).

Summarizing the results of crosses involving selections 1099, 368, 1083, and 475, it may be pointed out that seed softening in the F_1 plants, under the conditions of this experiment, either closely approached that of the "softer" parent or was intermediate between that of the two parental selections. Seed softening of the F_2 progenies usually ranged from that found in the "soft-shelled" parent to that found in the "hard-shelled" parent, with the majority of plans exhibiting all possible degrees of variation between the two extreme parental types.

CROSSES INVOLVING SELECTION 1130

Somewhat different results were obtained in crosses involving selection 1130.

There were three F_1 plants available from 1083×1130 crosses (fig. 2, *C*). The softening of the seed from these plants was considerably slower than that of either parental selection, as may be seen from the duration of the germination test. In the F_2 there were 26 plants available from the 1083×1130 cross (fig. 2, *C*), as well as 22 plants from the reciprocal cross. In both populations the rate of seed softening varied from very fast, even faster than that of seed of "soft-shelled" selections 1099 and 368, to very slow, even slower than that of either parent or than that of the F_1 plants from crosses between them.

Seed softening in 9 F_1 plants from the 1130×475 and reciprocal crosses was very slow and closely resembled that of the 475 parent (fig. 5, *B*). Thirty-one F_2 plants from the 1130×475 cross (fig. 5, *C*), as well as 34 F_2 plants from the reciprocal crosses were available. In both populations, the seeds ranged from a very fast softening type to a very slow softening type, with possibly a few individuals softening even more slowly than the 475 parent.

The results of the cross of 1130 with the soft-shelled selection 368 is somewhat different from those found in the crosses described above. In the three F_1 plants from the 368×1130 cross seed softening was intermediate between that of the two parental selections (fig. 3, *B*). For the first 15 days of the germination test the slope of the seed-softening curves for these F_1 plants resembled that of the 368 parent, but after 15 days it approached that of the 1130 parent. There were only 20 F_2 plants available from the 368×1130 cross (fig. 3, *C*). Seed softening in these plants ranged from that of the fast softening 368 parent to very slow softening, even slower than the 1130 parent. It is to be noted that in this F_2 population the extremely soft types, such as were found in the crosses of 1130 with 1083 and 475, were not observed.

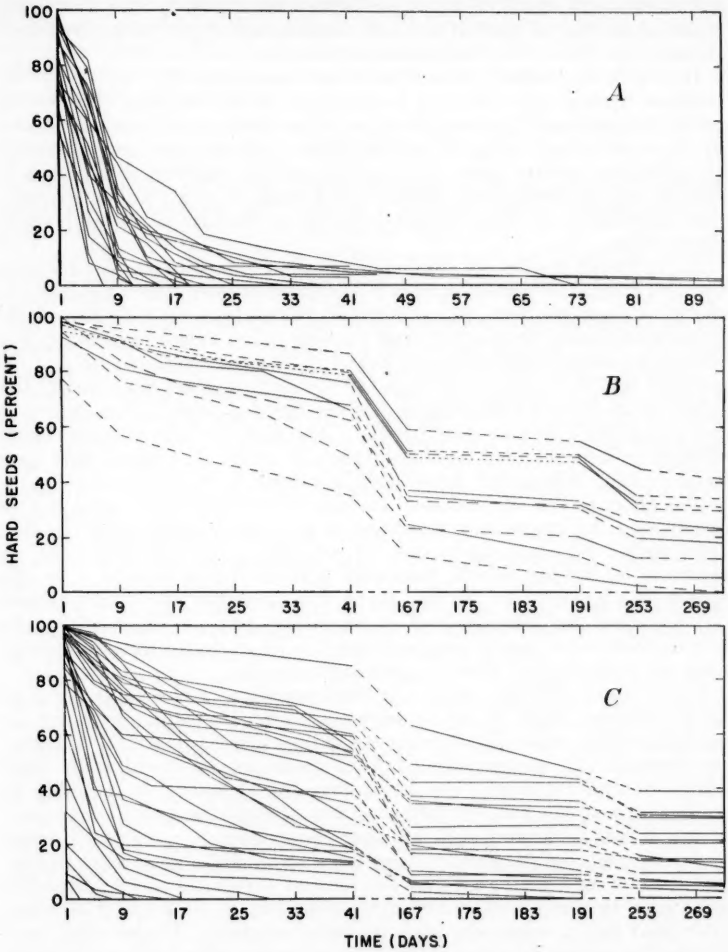

FIGURE 5.—Percentage of hard seeds on successive days of tests. *A*, F_2 from 1083×368 crosses; *B*, F_1 from 475×1130 (solid lines) and reciprocal (broken lines) crosses; *C*, F_2 from 1130×475 crosses.

DISCUSSION

Woodworth [6] made a cross between a soft-shelled and a hard-shelled variety of soybean, and soaked 25 of the F_1 seeds and 10 seeds from each of the F_2 plants in water for 8 days. He found soft-seededness (ability to take up water rapidly) to be partially dominant over hard-

[6] WOODWORTH, C. M. GENETICS OF THE SOYBEAN. Amer. Soc. Agron. Jour. 25: 36–51, Illus. 1933.

seededness. In the F_2 the seed softening varied from very fast, even faster than that of seed of the soft-shelled variety, to very slow, even slower than that of the hard-shelled parent.

In previous studies [7] the writer found that there were inherent differences among pure lines of beans, both in the amount of so-called hard seed produced under a given set of environmental conditions, and in the rates of softening of the seed coat in these impermeable seeds. By selecting several pure lines which exhibit marked differences in rate of seed softening and intercrossing them, it was possible to study the inheritance of this character which is fundamentally a physiological one.

As in other studies of the inheritance of developmental processes and quantitative characters,[8] the inheritance of seed softening does not exhibit a clear-cut segregation in the F_2 population but is rather a continuous variation from a very fast to a very slow rate of softening, the extremes often exceeding those found in the parental selections. No attempt will be made to evaluate the number of genes involved in the inheritance of seed softening as the segregating populations were too small to permit definite conclusions. The fact that the parental types of seed softening often were recovered in small F_2 populations does indicate that only a few genes were involved in a given cross.

The effect of the environment on hard-seed production must not be overlooked, for the results described in this study could be duplicated only under similar conditions. Changing either environment or methods of drying would probably give different results. As the writer has previously shown, there are probably two independent physiological factors involved in hard-seed production, namely, the development of a certain proportion of hard seed and the differential rates of softening of these impermeable seeds.

Under the conditions of the present experiment the proportion of hard seeds was high in all six selections, and, therefore, only the inheritance of the rate of softening could be studied. Under a different environment the proportion of hard seeds would be high in some selections and relatively low in others, and it would be possible to study not only the rate of softening but also the range of occurrence of hard seeds. Under still another environment, neither of these processes could be studied. There were small differences in the number of hard seed produced and the rate of softening of impermeable seeds even between such contrasting selections as 1099 and 475, when their seed had a relatively high moisture content. Under such conditions the segregating populations from such a cross are very similar. In the light of these facts it appears that hard-seed production in beans or other legumes offers an exceedingly favorable means of studying the inheritance of physiological reactions in plants.

It is evident that breeding offers the best approach to the problem of eliminating hard-seededness. In a previous study and in the present report, it has been shown conclusively that hard-seededness in beans is heritable. Although there is no detailed factorial analysis, the writer's study indicates that only a few genes are involved in the

[7] See footnote 2, p. 205.

[8] SMITH, H. H. RECENT STUDIES ON INHERITANCE OF QUANTITATIVE CHARACTERS IN PLANTS. Bot. Rev. 10: 349–382. 1944.

differentiation of hard and- soft-seeded selections. Therefore, in a breeding program with legumes an effort should be made to avoid using hard-seeded genotypes, particularly the slow-softening types. If soft-seeded strains are not available, they can be obtained from crosses between hard-seeded ones.

SUMMARY

Five selections of beans which exhibited marked differences under certain environmental conditions in regard to the rate of softening of hard seeds were intercrossed in various ways.

The plants of the original selections, as well as F_1 and F_2 plants from crosses between these selections, were grown together in a single environment.

After harvesting, seeds from these plants were first stored in the laboratory and then were kept for 5 days in an electric drier, in which the moisture content of the seed was reduced to about 6.61 percent.

Upon removal from the drier, the seed was placed in a germinator and records were kept of hard and swollen seeds throughout the test. Under these conditions the two "soft-shelled" selections were unchanged and seed softening was like that reported in previous studies, while in three "hard-shelled" selections seed softening was even more prolonged.

Seed softening in various F_1 crosses either approached closely that of the fast-softening parent or was intermediate between that of the two parental selections.

In the F_2 seed softening ranged between that found in the two contrasting parental selections, often with practically all possible degrees of variation between these two extremes represented.

Seed of the F_1 crosses involving selection 1130 were extremely slow in softening. The F_2 seed exceeded in both rapidity and slowness the rates of softening in the two contrasting parental types; that is, some softened faster than the fast-softening parent, and others softened more slowly than the slow-softening parent, the majority softening at variously intermediate rates.

In the progenies other than those of the 1130 crosses, the parental extremes were not exceeded but were usually recovered. Since F_2 populations were small only a few genes appear to be involved.

THE RELATION BETWEEN AGE, NUMBER, AND TYPES OF CELLS IN THE PERIPHERAL CIRCULATION OF CHICKEN EMBRYOS UNDER NORMAL AND EXPERIMENTAL CONDITIONS[1]

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INTRODUCTION

An extensive review and summary of the literature on natural transmission of the avian leukosis complex in the fowl has been made by Jungheer (*13*).[3] At one time or another three routes of transmission have been considered by workers in this field; namely, (1) through the egg, (2) by contact, and (3) by mechanical vectors such as insects. The review shows, however, that the mode of transmission of this disease is still open to question.

Waters and Prickett (*22*) and Waters (*21*) report that chickens hatched from eggs of infected dams, reared in complete isolation under rigid quarantine, showed an incidence of lymphomatosis of 16 percent, while other progeny from disease-free dams, reared in isolation and likewise quarantined, showed no lymphomatosis. On the basis of these observations, these authors suggest that lymphomatosis may be an egg-borne disease.

If the disease is egg-borne, quantitative, or qualitative cytological differences of such magnitude may be present in the peripheral circulation of embryos from lines of chickens which show various degrees of resistance and susceptibility to lymphomatosis as to make positive identification of the disease possible. It is well known that pronounced cytological variations are found occasionally in the peripheral blood of adult chickens infected with lymphomatosis (*1*, *10*, *13*).

The work on the developmental and genealogical history of the various cell types in the hemopoietic foci of the chicken embryo has been ably reviewed by several authors (*5*, *7*, *9*, *20*). However,

[1] Received for publication April 12, 1946. Journal article No. 806 (n. s.) of the Michigan Agricultural Experiment Station.

[2] The writer is indebted to Berley Winton, director, and to staff members of the U. S. Regional Poultry Research Laboratory for a grant-in-aid and for the use of laboratory facilities; to Dr. N. F. Waters, geneticist, for making available the hatching eggs from inbred chickens resistant and susceptible to lymphomatosis; and to Dr. B. R. Burmester, physiologist, for supplying the RPL–17 tumor mince; and to Dr. C. O. Prickett, pathologist, for taking the photomicrographs shown in figure 2.

[3] Italic numbers in parentheses refer to Literature Cited, p. 238.

only two reports, those of Sugiyama (*20*) and Dawson (*8*), have been primarily concerned with the relative numbers of the various cell types in the peripheral circulation of chicken embryos. Dawson based his conclusions on the study of only a limited number of specimens. Furthermore, he used the terminology of the unitarian hematologists while Sugiyama used that of the so-called dualistic hematologists. For this reason it was difficult to correlate the results given in the two papers. It therefore seemed advisable to make a differential count of the various cell types in the peripheral circulation of the chicken embryo to ascertain the "normal" frequency of occurrence of the cell types in the peripheral circulation in embryos of various ages.

The objectives of the present study were twofold: (1) To identify and ascertain the mean percentages of the various cell types in the peripheral circulation of inbred embryos of various ages from lines of chickens which showed various degrees of resistance and susceptibility to lymphomatosis; and (2) to study the effects produced on the peripheral blood of embryos by the injection of both normal and pathological tissues.

MATERIALS AND METHODS

The embryos used in this study were from lines of Single-Comb White Leghorn stock which showed various degrees of resistance to lymphomatosis (*42*). All embryos carry the number of the dam.

The majority of the blood samples used in this study were obtained from the heart with a micropipette. Two hundred or more embryos were studied by the supravital methods of Cunningham and Tompkins (*3*). An additional 8 to 20 duplicate samples of blood were obtained for each day of incubation from 48 hours until the day of hatching, and then they were stained with Wright and May-Grünwald Giemsa stains. All blood samples were examined, and preliminary differential counts of the cells of several fields in each of the blood smears were made to ascertain the extent of variation for each age. Blood samples which seemed to show all cytological variations for a given age were then selected for differential counts. From 4,000 to 11,000 cells were counted for each age group. Counts were made at 72-hour intervals. The mean diameters of cells and nuclei were ascertained by making 25 measurements with a Zeiss screw eyepiece micrometer.

OBSERVATIONS

EMBRYOS OF 71–76 HOURS' INCUBATION

Variations in Cell Types

Table 1 and figures 1 and 2 show that two generations of red blood cells, the primitive and definitive, are present in the peripheral circulation of the chicken embryo. The photomicrographs in figure 2, *A*, *B*, and *C*, reveal that the peripheral circulation of embryos of 71 to 76 hours' incubation contain primitive erythroblasts and hemocytoblasts. In embryos F1009 and F1043, the large primitive erythroblasts with medium hemoglobin had a mean diameter of 12.91μ and 11.39μ, respectively. The nucleus in the former had a diameter of 7.28μ, while that of the latter was 8.03μ (Wright stain). In vital

preparations the cytoplasm of these cells (fig. 1, *G*) had a yellowish tinge, and in most preparations a rosette of neutral red bodies could be identified in the cytoplasm midway between the nucleus and cell wall. Figure 1 shows that small particles of chromatin were present in the nucleus. Preparations that were left on a warm stage at 34.5° for 4 to 6 hours frequently contained some erythroblasts with blebs on the surface; these blebs in many instances pulled away from the cell and became free. The primitive erythroblast has been recognized by many writers as the first hemoglobin-containing cell of the vertebrate embryo.

TABLE 1.—*Mean percentage of cells of various types in the peripheral blood of embryos of various ages*

Embryo number	Age	Cells counted	Primitive erythroblasts		Primitive erythrocytes	Definitive erythroblasts		Definitive erythrocytes	Hemocytoblasts	Thrombocytes	Degenerating cells
			Medium hemoglobin	Much hemoglobin		Little hemoglobin	Medium hemoglobin				
	Hours	*Number*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
F1009 F1029	71–76	3,310	96.64	0	0	0	0	0	1.74	0	1.27
F1029 F1040											
F1043, F1050											
F1051											
E453, E608	93–100	4,100	95.50	0	0	0	0	0	1.96	0	2.51
E680, E701											
F1051, F1056, F1460–2											
E190, E213, E347, E348, E635, E691, E799 F1043	139–148	5,172	0	66.11	.40	4.85	12.43	10.61	.58	0	4.91
E190, E321, E440, E605, E609, E797, E814, E882	213–217	6,300	0	5.14	1.85	.14	1.74	90.22	.03	0	.85
50, E894–1, E888–1, E894–3, E888–2, E894–3, (2) E888–3, E894–5	283–288	6,200	0	6.59	0	.13	3.72	88.29	0	.09	1.01
E888–2, E893–2, E903–1, E894–6, E907–1, E897–3, E907–2	356–360	6,000	0	2.65	.03	.03	.91	95.38	0	.21	.55
E881–1, E888–3, E894–4, E1460–1, E1460–3	425–431	5,600	0	.09	0	0	0	99.16	0	.68	.03
E898–1, E905–4, E905–1, E907–4, E907–2	475–480	5,000	0	.06	0	0	.06	98.52	.02	1.24	0

In embryo F1009 primitive erythroblasts with medium hemoglobin had a mean diameter of 9.84μ, and in embryo F1043 (71–76 hours' incubation), the mean diameter was 8.11μ. These primitive erythroblasts appeared to be derived from the large erythroblasts since mitotic division reached 0.39 percent, and in the telophase of these divisions the diameter of the daughter cells approximated that of the medium-sized erythroblasts. In supravital preparations a portion of the large primitive erythroblasts appeared to divide atypically to form medium-sized erythroblasts.

Figure 2, *A* and *B*, shows hemocytoblasts of various sizes found in the peripheral circulation of embryos of 71–76 hours' incubation. The largest of the hemocytoblasts correspond to the lymphocytes of Dantschakoff (4, 5, 6, 7). This author believed that such lymphocytes were morphologically, as well as functionally, essentially the same as

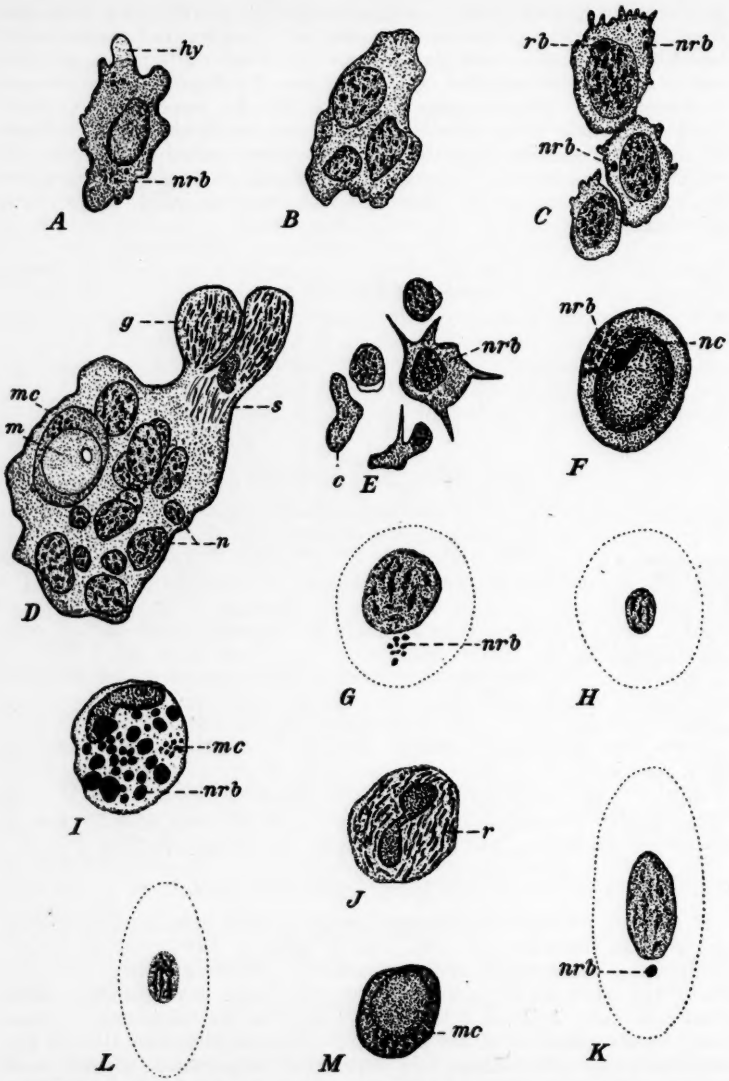

FIGURE 1.—Camera lucida drawings of peripheral blood (supravitally stained with neutral red and Janus green) of chicken embryos of various ages: *A*, Hemocytoblast, 71–74 hours' incubation; *B*, multinucleated hemocytoblast, 93–96 hours' incubation; *C*, fragmentation of a hemocytoblast to produce thrombocytelike cells; *D*, giant cells, 257–260 hours' incubation, showing granulocyte differentiating from surface; *E*, fragmentation of giant cell to produce thrombocytelike cells, 285–288 hours' incubation; *F*, transitional hemocytoblast (primitive erythroblast), 117–120 hours' incubation; G, primitive erythro-

the lymphocyte of the adult chicken. The smallest hemocytoblast identified seemed to agree morphologically with the dwarf lymphocyte of Dantschakoff.

The hemocytoblast in the peripheral circulation of embryos of 71–76 hours' incubation, exhibited in Wright-stained preparations a pronounced basophilic cytoplasm which formed blebs that were frequently separated from the cell (fig. 2, *A*), leaving the nucleus naked or almost so at the point of separation. Not all hemocytoblasts, however, showed this tendency to form blebs; in fact, bleb formation seemed to be an artifact since this phenomenon was not seen in fresh vital preparations. The nucleus was large, often reaching a mean diameter of 8.79μ. Frequently, the nucleus seemed to be empty, and when this was the case it exhibited a weak basophilia; at other times, the small dustlike chromatin particles were uniformly distributed, and gave to the nucleus a light, sky-blue, stippled appearance. One or more nucleoli were frequently seen and these were usually a darker, robin's-egg blue.

In vital preparations the cytoplasm of the hemocytoblast was colorless and had a ground-glass appearance (figs. 1, *A* and *B*). A variable number of neutral red bodies were sometimes observed in the cytoplasm. Pseudopod formation was rather common, and at the distal tips of the pseudopods a hyaline cap was evident in some specimens. One or more nucleoli were frequently found in the nucleus. A refractive body was sometimes seen in the cytoplasm in about the same position in the cell as the centrosome in stained preparations.

Table 1 shows that the mean percentage of degenerating cells in embryos incubated for 71–76 hours was 1.27. These cells occurred singly or in clumps of three, four, or more. The cytoplasm was either weakly basophilic, acidophilic, or neutrophilic and formed a narrow, irregular border around the nucleus. Some nuclei were naked, the chromatin within the nuclei had undergone a pronounced condensation, and its affinity for basic dyes had increased; i. e., the nuclei showed a pronounced pyknosis. In some blood smears the nuclei resembled those of small hemocytoblasts; in others they looked more like those of erythroblasts. In one clump of six cells, the largest nucleus in the group had a diameter of 5.75μ and the smallest, 3.07μ.

Variations in Number of Cell Types in Individual Blood Samples

The number of primitive erythroblasts counted in each of the smears used for arriving at the mean percentage given in table 1 for embryos of 71–76 hours' incubation were in fairly close agreement. The varia-

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blasts with medium hemoglobin, 91–94 hours' incubation; *H*, primitive erythroblast (orthochromatic) 141–144 hours' incubation; *I*, myelocyte, 285–288 hours incubation; *J*, polymorphonuclear heterophilic granulocyte, 284–287 hours' incubation; *K*, primitive erythrocyte, 141–144 hours' incubation; *L*, definitive erythrocyte, 141–144 hours' incubation; *M*, hemocytoblast 6 hours after hatching. × ¼.

c, Cytoplasm; *g*, polymorphonuclear heterophilic granulocyte; *hy*, hyaline layer; *m*, hemocytoblast; *mc*, mitochondria; *n*, nuclei; *nc*, nucleolus; *nrb*, neutral red body; *r*, spindle-shaped crystalline bodies; *rb*, refractive body; *s*, folds. Bausch and Lomb, 2-mm. apochromatic objective with compensating ocular 10 was used. Drawings made at 1.8 cm. above table level.

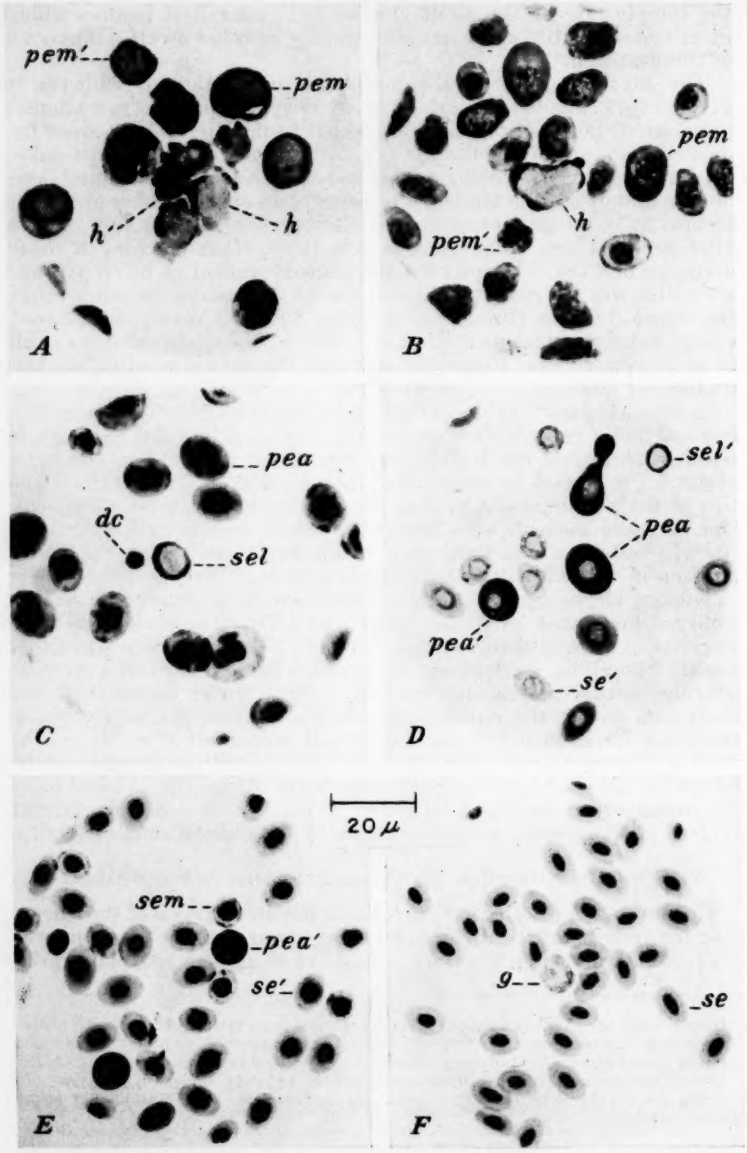

FIGURE 2.—Photomicrographs of peripheral blood (May-Grünwald Giemsa stain) of chicken embryos of various ages: *A*, 69–72 hours' incubation; *B*, 97–100 hours' incubation; *C*, 145–148 hours' incubation; *D*, 284–287 hours'

tions observed in number of hemocytoblasts (small, medium, and large) were more striking. In embryos from susceptible line 15, a maximum of 21 hemocytoblasts was found in F1050 (500 cells counted), and a minimum of 3 in F1029 (300 cells counted). In susceptible embryo F1040 from line 2, 11 hemocytoblasts were found among the 410 cells counted.

In blood smears from embryos F1050, F1029 (line 15), and F1040 (line 2) one large cell with a pronounced vacuolization of the cytoplasm was observed in each of the three slides. The morphological features of this cell agreed fairly closely with the description given by Murray (*16*) for wandering cells.

On the basis of the observations made in this study and also from the descriptions given in the literature, it seems fairly safe to conclude that the vacuolated cells found in the peripheral circulation were the histiocytes of other authors.

EMBRYOS OF 93-100 HOURS' INCUBATION

Variations in Cell Types

The primitive erythroblasts decreased from a mean percentage of 96.64 in embryos of 71–76 hours' to 95.50 in embryos of 93–100 hours' incubation (table 1). Proliferation must have been restricted mainly to the largest primitive erythroblasts since percentage mitosis (93–100 hours) was found to be 0.62. The hemocytoblasts (lymphocytes) increased from a total mean percentage of 1.74 in embryos of 71–76 hours to 1.96 in embryos of 93–100 hours and, correspondingly, the degenerating cells increased from 1.27 to 2.51. Histiocytes were not identified in the latter and a further check was made by examining 150 fields in each slide prepared from embryos of 93–100 hours' incubation, but the results were negative.

Variations in Number of Cell Types in Individual Blood Samples

All differential counts of cells in the embryos of 93 to 100 hours' incubation showed that the number of large erythroblasts was greater than the number of medium erythroblasts. Only 1 embryo (E701, line 12) from the inbred resistant lines was used for experimentation. This embryo showed only 1 hemocytoblast in a differential count of 500 cells, whereas the embryos from susceptible lines (4 from line 15, 2 from line 8) showed considerable variation in total number of hemocytoblasts. The highest number of hemocytoblasts (31 in 500 cells) was found in F1051, susceptible line 15, and the next highest number in F1460, embryo 2, susceptible line 15. The highest number of

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incubation; *E*, 356–359 hours' incubation; *F*, 429–431 hours' incubation. All × 700.

dc, Degenerating cell; *g*, polymorphonuclear heterophilic granulocyte; *h*, large hemocytoblast; *h'*, medium-sized hemocytoblast; *pea*, large primitive orthochromatic erythroblast; *pea'*, medium-sized primitive orthochromatic erythroblast; *pem*, large primitive erythroblast with medium hemoglobin; *pem'*, medium-sized primitive erythroblast with medium hemoglobin; *se*, definitive erythrocyte; *se'*, immature definitive erythrocyte; *sel*, definitive erythroblast with little hemoglobin; *sel'*, small definitive erythroblast with little hemoglobin; *sem*, definitive erythroblast with medium hemoglobin.

degenerating cells (22) was found in E701, resistant line 12, and the lowest in E608 and E680, susceptible lines 15 and 8 respectively.

EMBRYOS OF 139–148 HOURS' INCUBATION

Variations in Cell Types

The primitive erythroblasts with a medium amount of hemoglobin decreased to zero and the primitive erythroblasts with much hemoglobin (dark rose red in fixed preparations) increased from zero in embryos of 93–100 hours' incubation to a total mean percentage of about 66 in embryos of 139 to 148 hours' incubation (table 1). Dantschakoff (*5*) found that the primitive erythroblasts (orthochromatic) reached complete maturity on the fifth day of incubation and that at maturity the cytoplasm of the cells in fixed preparations (celloidin technique) stained pure red, the nucleus remained small, apparently shriveled, the nucleus became less clear, and mitotic activity ceased. Measurements made in this study showed that the large primitive erythroblasts with much hemoglobin had a mean cell diameter of 13.16μ and that the diameter of the nucleus was 4.69μ. Primitive erythroblasts of medium size with much hemoglobin had a cell diameter of 10.19μ and a nuclear diameter of 3.89μ, while the diameters of the cell and nucleus decreased to 8.73μ and 3.66μ, respectively, in the smallest erythroblast of this type.

Table 1 shows that the mean percentage of definitive erythroblasts with little hemoglobin was 4.85. In embryos E348 and E635, the diameters of the cell were, respectively, 9.55μ and 9.13μ, and those of the nucleus 7.18μ and 7.00μ.

Figure 1, *D*, shows a supravital preparation of the youngest cell in the definitive erythroblast series. This cell (*m*) was connected with the giant cells by a protoplasmic bridge. A comparison of this cell with the lymphocyte of the adult chicken (figure 1, *M*) shows the two to be similar in that the cytoplasm of both had a ground-glass appearance with oval or round mitochondria and limited amounts of chromatin in the nuclei. Wright-stained definitive erythroblasts with little hemoglobin likewise resembled lymphocytes (hemocytoblasts) of adult blood.

Table 1 shows that the definitive erythroblasts with medium hemoglobin (polychromatic) increased from zero in embryos of 93–100 hours to a mean percentage of 12.43μ embryos of 139–148 hours' incubation. The mean diameters of this cell (embryo E635) and its nucleus were 8.22μ and 4.68μ, respectively. It was evident in all slides that this cell was derived from the definitive erythroblasts with little hemoglobin, since transitional stages could be arranged in an orderly sequence from immature erythroblasts to more mature erythroblasts.

Variations in Number of Cell Types in Individual Blood Samples

An analysis of individual counts of cells found in the peripheral circulation of the embryos incubated for 139 to 148 hours showed that the numbers of primitive and definitive erythroblasts used for obtaining the mean percentages in table 1 were approximately the same in resistant embryos E213 (line 6), E348 (line 6), and E635 (line 10), and that in resistant embryo E799 (line 13) the definitive erythroblasts

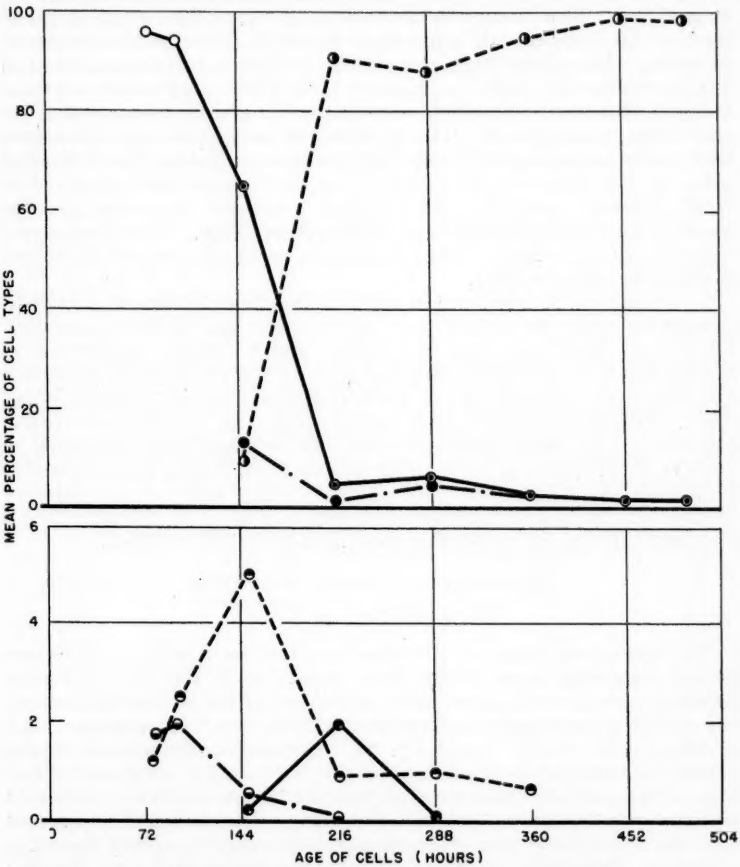

FIGURE 3.—Relation between age and mean percentage of cell types in the peripheral circulation of chicken embryos of various ages.

○=Primitive erythroblasts with medium homoglobin (polychromatic);
◉=primitive erythroblasts with much hemoglobin (orthochromatic);
◑=definitive erythrocytes;
●=definitive erythroblasts with medium homoglobin (polychromatic);
◓=degenerating cells;
◒=hemocytoblasts; and
◔=primitive erythrocytes.

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with little hemoglobin decreased to zero, while the definitive erythrocytes increased to 70 percent; consequently, the mean percentage for definitive erythrocytes given in table 1 is 10.61. Other striking variations in numbers of cell types were found in this resistant group of embryos. One of the highest numbers (687 in a differential count of 846) and also the lowest number (101 in 700) of primitive erythroblasts with much hemoglobin were found in resistant embryos E213 and E799, respectively. The number of hemocytoblasts remained fairly constant; a high of 5 cells in 797 cells was obtained for E635 and a low of 1 in 846 cells for E213. Four myelocytes were observed in E348 (409 cells counted). The smallest number of degenerating cells, 8 and 9, were found in E635 and E799, respectively. Four histiocytes were identified in 2 of 8 slides; 2 each in resistant embryo E190 and susceptible embryo E691.

A comparison of cell types in the peripheral circulation of individual susceptible embryos showed that the primitive and definitive erythroblasts were fairly constant in number, and a further comparison of susceptible with resistant embryos showed the numbers of primitive and definitive erythroblasts in the two groups to be in essential agreement. Other variations were noted, however, in the susceptible embryos. Ten hemocytoblasts (626 cells counted) were identified in embryo E347 (line 7), 2 (556 cells counted) in F1043 (line 7), and 6 (597 cells) in E691 (line 15). Degenerating cells were relatively abundant. Fifty-five degenerating cells in a total of 597 were found in embryo E691, 54 in 556 in F1043, and 50 in 626 in E347.

EMBRYOS OF 213–217 HOURS' INCUBATION

Variations in Cell Types

The mean percentage of primitive erythroblasts with much hemoglobin decreased from about 66 in embryos of 139 to 148 hours' incubation to about 5 in embryos of 213 to 217 hours' incubation and the primitive erythrocytes increased from a mean percentage of 0.4 to about 1.85 (table 1 and fig. 3). The mean dimensions of the primitive erythrocyte in embryo E797 were 12.74μ long and 8.95μ wide with a nuclear diameter of 4.18μ. In Wright stain the primitive erythrocytes become a dark rose-red color. Dantschakoff (*5*) states that the primitive erythrocytes cannot be sharply separated from the primitive erythroblasts. She believes that degeneration of this so-called provisional red cell begins on the fourth or fifth day and is characterized by the nucleus becoming smaller, more shrunken, and paler.

The definitive erythroblasts with little hemoglobin decreased from a mean percentage of 4.85 in embryos of 139–148 hours to 0.14 in embryos of 213–217 hours, and there was a corresponding decrease in definitive erythroblasts with medium hemoglobin from 12.43 to 1.74. The definitive erythrocytes, however, increased from a mean percentage of 10.61 to 90.22. The definitive erythrocytes were smaller than the primitive erythrocytes and the affinity of their cytoplasm for eosin stain was less pronounced. The mean length of the definitive erythrocytes was 10.32μ; width, 7.00μ; diameter of nucleus, 4.29μ. Considerable variation was found in their staining reaction. Some stained grayish blue with a suggestion of pink; others were poly-

chromatic; and still others, the more mature ones, were light rose red. The affinity for eosin was never so pronounced in the definitive erythrocytes of either the embryo or adult as it was in the primitive erythrocytes. These differences in staining reaction and also in size were used as criteria for the identification of primitive and definitive erythrocytes. Hall (*12*) found that the affinity of hemoglobin for oxygen decreased in the chick embryo from the tenth day up to 3 weeks after hatching. Hemoglobin of early development, according to Hall, was replaced by a hemoglobin late in development which had less affinity for oxygen.

Variations in Number of Cell Types in Individual Blood Samples

In the group of 213–217 hours' incubation, three embryos (E190, E440, E797) were from resistant line 13 and one (E882) from resistant line 10. A comparison of the data obtained by differential counts of cells in the peripheral blood of all resistant embryos showed that the frequency of occurrence of both erythroblasts (primitive and definitive) and hemocytoblasts was essentially the same in all the embryos. There was likewise little variation in the number of degenerating cells.

The embryos E321, E609, and E814 were from susceptible line 15, and E605 was from susceptible line 7. The differential counts showed that cell types in individual embryos of both lines 15 and 7 were fairly constant in number, and a comparison of the individual differential counts of the susceptible embryos with those of the resistant embryos showed no significant qualitative or quantitative differences.

EMBRYOS OF 283–288 HOURS' INCUBATION

Variations in Cell Types

In embryos of 283–288 hours' incubation there were no primitive erythroblasts with medium hemoglobin, and the increase in primitive erythroblasts with much hemoglobin over the preceding age group was less than 2 percent (table 1).

The definitive series of red cells show similar variations in number. Erythroblasts with medium hemoglobin increased from a mean percentage of 1.74 to 3.72, and the definitive erythrocytes decreased from 90.22 to 88.29. These variations were not considered significant.

For the first time in this study thrombocytes appeared. Observations on vital preparations of peripheral blood of embryos of various ages revealed giant cells (fig. 1, *D*) with 15 or more nuclei. These may correspond to the clumps of hemocytoblastlike cells in Sugiyama's stained preparations which were alleged to demonstrate thrombogenesis. Some of the nuclei in the giant cells, at least the smaller, more pyknotic ones, may be ingested red cells, since on numerous occasions red cells in vital preparations were observed in the process of being phagocytized. When the giant cells were maintained on a warm stage, bleblike structures frequently developed on the surface and sometimes these contained numerous cytoplasmic rod-shaped granules, with or without nuclei. In one vital preparation, rod-shaped granules were observed to collect in and around a kidney-shaped nucleus, and at this point there was no indication of a bleb; this was interpreted as an early stage in granulocyte forma-

tion. Observations made on 200 or more vital preparations lead the writer to believe that these giant multinucleated cells arose by fusion of stem cells which were identified by hemocytoblasts, and also by atypical nuclear division.

After the clumping of individual hemocytoblastlike cells, the cellular membranes seemed to disintegrate at the points of contact with other cells, and only that part of the membrane which was free and not in contact with other cells, was left intact. The resultant cells seemed to retain hemopoietic potentialities, and for this reason differentiation of granulocytes appeared to take place under the stimulus of unfavorable environmental factors (fig. 1, *C*).

Obviously blood samples, sealed with vaseline, on a warm stage are in an environment very different from that of the living animal. Under such conditions immature cells with fragile cell membranes probably disintegrate to produce extracts, and extracts may accelerate metabolic activity and facilitate differentiation of granulocytes. The writer (*11*) has found that extracts of paramecia facilitate attachment and ingestion in the large mutinucleated rhizopod *Pelomyxa carolinensis*. The production of extracts in blood cultures was probably facilitated by the multinucleated cells themselves. Erythroblasts in contact with giant cells were frequently observed to lose their hemoglobin in the space of 1 or 2 seconds. Other erythroblasts, further removed, were normal in every respect. Thus it would appear that some material in the vicinity of the giant cell altered the cell membrances of erythroblasts and allowed the hemoglobin to escape.

The giant cells sometimes exhibited other developmental potentialities. If they were left at room temperature on the stage of a microscope, fragmentation frequently occurred. The resultant cells showed pyknotic nuclei with a ground-glass, metabolic cytoplasm, (fig. 1, *C* and *E*). Neutral red bodies were frequently found in the cytoplasm. Cells produced in this manner were similar morphologically to the thrombocytes. This cytological process in the giant cells was similar to fragmentation observed by the writer in the large multinucleated rhizopod *Pelomyxa carolinensis* when it was subjected to temperatures of 4° or 45°.[4]

Other observations made by the writer suggest that thrombocytes may be closely related to young erythroblasts. Stained preparations frequently showed round and elliptical thrombocytes with a stain-free, weakly acidophilic or basophilic, cytoplasm, and pyknotic nuclei. Eosin-staining granules were frequently found in the vicinity of the nucleus in the same relative positions as the neutral red bodies in supravitally stained preparations of erythroblasts and erythrocytes. Both in amount and pattern the chromatin in thrombocytes showed a pronounced similarity to that in young erythorcytes. Blount (*1*) believed that the thrombocyte was an erythrocyte in which the hemoglobin did not proceed to maturity. Because of these degenerative phenomena, the cytoplasm lost its characteristic polychromatic-eosinophilic property to stain faintly basophilic. For other views in regard to the origin of the thrombocytes Mjassojedoff (*14*) and Sugiyama (*20*) should be consulted.

[4] Unpublished data.

Variations in Number of Cell Types in Individual Blood Samples

A review of individual differential counts made from the peripheral blood of embryos from susceptible line 15 showed the blood picture to be fairly constant in all embryos. Only one hemocytoblast was observed; this was found in embryo 50 (line not recorded). One polymorphonuclear heterophilic granulocyte was found in 3 of the 10 differential counts. The maximum number of 5 thrombocytes was found in E894–3 and a minimum of 1 in E894-3 (embryo 2). Thrombocytes were not found in other slides, although 600 or more cells were used for each differential count. Only 2 slides, 50 and E888–2, showed definitive erythroblasts with little hemoglobin; the maximum, 7, was found in 50 and the minimum, 1, in E888–2. A maximum of 23 degenerating cells was counted in E888–1 and a minimum of 3 in E894–1; in 3 of the 9 differential counts no degenerating cells were observed.

EMBRYOS OF 356–360 HOURS' INCUBATION

Variations in Cell Types

A comparison of mean percentages of cell types obtained for embryos of 283–288 hours' incubation with percentages obtained for 356–360 hours' incubation (table 1 and fig. 3) shows that the primitive erythroblasts with much hemoglobin decreased from 6.59 percent in the former to 2.65 percent in the latter, and the definitive erythroblasts with medium hemoglobin decreased from 3.72 percent to 0.91 percent. The definitive erythrocytes increased from 88.29 in embryos of 283–288 hours' incubation to 95.38 in embryos of 356–360 hours' incubation. Variations in size of definitive erythrocytes were also recorded. Immature definitive erythrocytes (fig. 2, *E*, *se'*) had a mean length of 10.32μ, a width of 7.00μ, and an oval nucleus of 4.27μ; mature erythrocytes had a length of 12.54μ and a width of 6.47μ, with a nucleus 4.85μ long and 2.48μ wide. Olson (*17*), after reviewing the literature, gave the consensus for erythrocyte length as 12.2μ and width as 7.3μ.

Variations in Number of Cell Types in Individual Blood Samples

All embryos used for experimentation in the 356–360 hour group were from susceptible line 15. No hemocytoblasts were found although 700–1,200 cells were counted in each of 8 slides. Similarly, other cell types had almost disappeared from the circulation. Only one embryo (E907–2) showed definitive erythroblasts with little hemoglobin (2 of 1,200 cells). In 3 of 8 slides definitive erythroblasts with medium hemoglobin persisted—a maximum of 37 in E893–2 (800 cells counted), 12 in E888–2 (600 cells), and a minimum of 6 in E907–2 (1,200 cells). Polymorphonuclear heterophilic granulocytes decreased. A maximum of 4 granulocytes was found in embryo E907–2 (1,200 cells counted) and a minimum of 2 in 3 of 6 slides, E893–2, E894–6, E907–1 (700 to 900 cells counted). No hemocytoblasts were identified in the peripheral circulation of the 7 embryos. Thrombocytes which showed the characteristic eosinophilic granules were identified in only 1 of the 7 slides.

EMBRYOS OF 425–431 HOURS' INCUBATION

The mean percentage of definite erythrocytes increased from 95.38 in embryos of 356–360 hours' incubation to 99.16 in embryos of 425–431 hours' incubation (table 1). Primitive erythroblasts with much hemoglobin decreased from a mean percentage of 2.65 to 0.09. No hemocytoblasts or heterophilic granulocytes were found in the peripheral circulation, although a total of 5,600 cells was counted in 5 slides. The mean percentage of thrombocytes increased from 0.21 to 0.68 in embryos of 425–431 hours' incubation. Thrombocytes were found in the peripheral circulation of all embryos. The number was fairly constant. A high count of 10 was recorded for embryo E894–4 and a low of 5 in embryo E1460–1. All embryos used for obtaining the mean percentages of cell types in this age group were from susceptible line 15.

EMBRYOS OF 475–480 HOURS' INCUBATION

With the exception of thrombocytes, the mean percentages of cell types in embryos of 425–431 hours' incubation and those of 475–480 hours' incubation were essentially the same. Thrombocytes increased from 0.68 in embryos of 425–431 hours' incubation to 1.24 in embryos of 475–478 hours' incubation. Two heterophilic granulocytes were found in E905–1, 3 in E907–2, and none in the remaining 3 embryos. All specimens in this incubation group were obtained from eggs of susceptible line 15.

It is apparent from the data so far presented that the number of blood cells in the peripheral circulation of embryos remains fairly constant from about the seventeenth day until the date of hatching and that the qualitative features of these cells closely approximate those of adult chickens.

MEAN PERCENTAGES OF CELLS IN THE PERIPHERAL CIRCULATION OF 7 AND 14-DAY INBRED SUSCEPTIBLE AND RESISTANT EMBRYOS

Embryos from inbred susceptible and resistant lines were used exclusively in the following experiments. Thirty-two embryos were obtained from hatching eggs collected from 10 hens from susceptible line 7. Fifteen percent of the breeding stock in this line developed lymphomatosis in 300 days and 23.3 percent in 600 days.

A total of 23 embryos was obtained from hatching eggs produced by 8 hens in resistant line 10. No lymphomatosis, either neutral or visceral, developed in this line in 600 days.

Nineteen embryos were obtained from hatching eggs produced by 7 hens in susceptible line 11. Breeding stock in this line showed 14.4 percent lymphomatosis in 300 days and 45.9 in 600 days.

A total of 44 embryos was obtained from eggs produced by 12 hens in resistant line 13. Breeding stock in this line showed 8.7 percent lymphomatosis in 300 days and 30.4 percent in 600 days.

All blood smears were obtained from the heart of the embryos. The entire liver was taken and prepared for histological study by fixation in Petrunkevitch (*18*) fixative. Haematoxylin and triosin were used for staining.

In embryos of 161–167 hours' incubation (table 2) the mean percentage of definitive erythrocytes was highest in resistant line 13

TABLE 2.—*Mean percentage of cells of various types in the peripheral blood of resistant and susceptible embryos incubated for 161–167 and 329–339 hours, from various lines of chickens*

Line No.	Embryos used	Experiment No.	Cells counted	Age of embryos	Primitive erythroblasts (much hemoglobin)	Primitive erythrocytes	Definitive erythroblasts		Definitive erythrocytes	Hemocytoblasts	Heterophilic granulocytes		Thrombocytes	Histiocytes	Myelocytes		Degenerating cells
							Little hemoglobin	Medium hemoglobin			Rods	Granules			Acidophilic	Basophilic	
	Number		*Number*	*Hours*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Line 7 (susceptible)	20	1	8,147	161–167	36.97	2.78	0.44	18.31	40.46	0.12	0.01	0	0	0	0.01	0.01	0.84
Line 10 (resistant)	8	1	4,281	161–167	30.75	.56	.37	1.30	65.85	.02	0	0	0	0	0	0	1.12
Line 11 (susceptible)	2	1	790	164–167	34.55	.25	.50	3.79	60.37	.25	0	0	0	.10	0	0	.10
Line 13 (resistant)	17	1	12,737	164–167	23.34	.30	.61	1.44	73.05	.40	.007	0	0	.05	0	0	.76
Line 7 (susceptible)	12	2	14,731	329–332	.95	.16	.01	.48	97.26	.03	.10	0	.96	.007	.007	0	.007
Line 10 (resistant)	15	2	15,313	329–337	.89	.06	.04	.26	97.71	.03	.04	.006	.86	0	.03	.03	0
Line 11 (susceptible)	17	2	19,337	329–338	.84	.02	.22	.86	97.19	.01	.06	.01	.74		.01	0	0
Line 13 (resistant)	27	2	37,723	329–339	.75	.04	.12	.36	97.24	.10	.09	.004	1.21	.004	0	.03	0

(73.05), next highest in resistant line 10 (65.85), lower in susceptible line 11 (60.37), and lowest in susceptible line 7 (40.46). A further comparison of cell types shows that the mean percentage of definitive erythroblasts with medium hemoglobin was highest (18.31) in the embryos of susceptible line 7 and lowest (1.30) in those of resistant line 10. It was shown earlier in this paper that the cells forming the transitional stages in the development of the erythroblast can be arranged in an orderly sequence with definitive erythroblasts with little hemoglobin at one end and mature definitive erythroblasts at the other. These data suggest that the development of hemopoietic tissue in susceptible line 7 embryos was inhibited to a greater degree than it was in embryos of resistant lines. What was the nature of this inhibition? Obviously, it may be genetic or it may be due to an agent that was transmitted from dam to embryo. Autopsy records show that the incidence of lymphomatosis in line 7 progeny was relatively high (15 percent in 300 days) and took place earlier in life than it did in the progeny from lines 13 and 10 (8.7 and 0, respectively). If this high incidence of lymphomatosis in adult birds was dependent on the introduction of an agent into the egg under natural conditions on or before ovulation, it would follow that further evidence could be obtained either for or against egg transmission by a study of hemopoietic tissues of older embryos from the various lines of chickens.

In embryos of susceptible line 7 incubated for 161–167 hours a total of 10 hemocytoblasts was found in 5 of 20 (8,147 cells counted) blood samples. In line 13 resistant embryos of the same age 52 hemocytoblasts were identified in the peripheral blood of 9 of 17 (12,737 cells counted) embryos. However, in line 10 embryos (161–167 hours' incubation), which were the most resistant of the embryos studied, hemocytoblasts were identified in only 2 of 8 blood slides (4,281 cells). Resistance to pullorum disease in young chicks (*19*) and to rat sarcoma in chicken embryos (*15*) has been shown to be rather closely correlated with increase in number of lymphocytes (hemocytoblasts). If the increase in number of hemocytoblasts in the peripheral circulation of embryos of line 13 was a true expression of resistance to lymphomatosis, it would be expected that an increased number of lymphocytes would be maintained in the peripheral circulation of older embryos.

An analysis of the cells in embryos of 329–332 hours' incubation shows that 2 hemocytoblasts were identified in 12 blood samples (14,731 cells counted) from susceptible line 7 embryos, while 6 of 27 smears taken from resistant line 13 embryos contained 37 hemocytoblasts (37,723 cells counted). Embryos from resistant line 10 showed 5 hemocytoblasts in 4 of 15 blood smears (15,313 cells counted), while 1 of the 17 smears made from susceptible line 11 embryos showed a total of 4 hemocytoblasts in 19,337 cells. It is evident from these data and also from data recorded earlier in the paper that the number of hemocytoblasts in some susceptible embryos was about equal to that in some resistant embryos. Obviously, some embryos in susceptible lines are more resistant to lymphomatosis than others or it would be impossible to maintain these lines. In the final analysis, the over-all picture of the differential counts suggests, but does not demonstrate, that resistance to lymphomatosis was expressed in the

embryonal peripheral blood by increased numbers of hemocytoblasts. Hemopoietic variations of this sort differ in many respects from those observed in the peripheral blood of adult chickens infected with naturally occurring lymphomatosis. When the disease developed spontaneously on the laboratory premises, the peripheral blood of some adult chickens showed increased numbers of immature cells of the red cell series while others showed a slight increase in myeloblasts.[5]

MEAN PERCENTAGES OF CELL TYPES IN THE PERIPHERAL CIRCULATION OF EMBRYOS FOLLOWING INJECTION OF THE YOLK SAC WITH TUMOR MINCE AND MINCED NORMAL LIVER

Burmester and Prickett (*2*) have described a highly malignant strain of tumor developed from naturally occurring avian lymphomatosis; this strain they refer to as RPL–17. In experiment 1 (table 3), 3 gm. of tumorous RPL–17 liver was obtained from donor G1463A under aseptic conditions. The liver was minced, filtered through sterile gauze into a tumor mince bottle containing 9 cc. of NaCl solution (8.5 gm. in 1,000 cc. of distilled water), and the bottle was sealed with a sterile rubber cover. The tumor mince used for inoculation was removed from the bottle with a sterile 24-gage needle and a 2-cc. sterile syringe. All eggs in experiment 1 were incubated for 7 days and then removed from the incubator and washed in dilute heparin solution. Just before inoculation the shell was ground thin at the equator by means of a motor-driven burr, so that the 24-gage needle could be thrust into the yolk sac for inoculating it with tumor mince. Eight eggs were inoculated in this manner with 0.05 cc. of strain 17 tumor mince and 3 with 0.05 cc. of NaCl solution. They were then returned to the incubator along with 15 controls and left for 7 days. On the fourteenth day tumor-mince-injected, NaCl-injected, and control eggs were removed from the incubator and opened. Blood smears and pieces of liver were obtained from individual embryos for further study. All embryos used in the following experiments were from susceptible line 15 with the exception of F1002, F1036, F1040, and F1043, which were from susceptible line 2. Wright and May-Grünwald Giemsa dyes were used for staining blood smears. The livers were fixed in Petrunkevitch (*18*) fixative and then stained with haematoxylin-triosin.

In experiment 2, the methods used for the injection of tumor (donor G1463A) and NaCl solution were essentially the same as those in experiment 1 except that in experiment 2 injections were made with RPL–17 tumor mince and also with NaCl solution about 24 hours before the eggs were left in the incubator. All eggs were incubated for 14 days before the embryos were sacrificed for the collection of blood smears and liver for histological study. In experiment 2, 11 eggs were injected with 0.05 cc. RPL–17 tumor mince, 11 with normal NaCl solution, and the shells of 3 were punctured but not inoculated. Controls were likewise incubated and studied along with the injected eggs.

In experiment 3 the procedure for inoculation was essentially the same as that already described. Twelve eggs were injected with 0.05 cc. of RPL–17 tumor mince (donor G1269D) and 14 eggs with 0.05 cc. of normal NaCl solution. All inoculations were made about

[5] Personal communication from E. M. Denington.

TABLE 3.—*Mean percentage of cells of various types in the peripheral blood of embryos incubated for 326–338 hours after the yolk sac had been inoculated with RPL–17 tumor mince, grossly normal liver, and normal saline solution*

Embryo No.	Experiment No.	Treatment [1]	Cells counted	Age of embryo	Primitive erythroblasts (much hemoglobin)	Primitive erythrocytes	Definitive erythroblasts		Definitive erythrocytes	Hemocytoblasts	Heterophilic granulocytes (rods)	Thrombocytes	Acidophilic myelocytes
							Little hemoglobin	Medium hemoglobin					
			Number	*Hours*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
F1009, F1017, F1028, F1052, F1056, F1017, F1029.	1	IT_1	6,600	326–335	0.54	0.15	0.01	2.11	97.16	0	0.03	0.57	0
F1040, F1043, F1045, F1009, F1017, F1028, F1029, F1056.		C	11,231	327–331	.57	.06	0	1.13	97.45	0	0	.77	0
F1028, F1040, F1048	2	IT	4,900	332–335	1.22	.16	0	.12	97.22	0	.04	1.18	0
F1002, F1009, F1040, F1051		S	5,087	332–336	.61	.02	0	.49	97.70	0	0	1.18	0
F1028, F1029, F1030, F1040, F1043, F1348		C	8,000	334–337	.66	.10	0	.30	97.77	0	.02	1.13	0
F1025, F1027, F1033	3	IT	5,100	331–334	.77	.04	0	.10	98.19	0	.06	.70	0
F1008, F1025, F1025, F1031, F1036		S	5,900	332–338	.35	0	0	.10	98.73	0	.06	.74	0
F1004, F1009, F1056–1	4	IL	5,297	327–330	2.35	1.62	.64	2.07	90.65	.32	.03	1.66	.60
F1009, F1025, F1056		C	7,000	327–331	.95	.10	0	.31	97.90	0	0	.70	.03

[1] IT_1, 0.05 cc. of RPL–17 tumor mince injected into yolk sac of embryos incubated for 7 days; C, uninjected embryos; IT, 0.05 cc. of RPL–17 tumor mince injected into yolk 24 hours before start of incubation; S, 0.05 cc. of saline solution injected 24 hours before start of incubation; IL, 0.05 cc. of grossly normal liver mince injected into yolk sac 24 hours before start of incubation.

24 hours before incubation. Likewise, all eggs were incubated for 14 days before the embryos were used for histological study.

The inoculum employed in experiment 4 was made from the liver of a chicken (donor G1368A) grossly negative for lymphomatosis, from susceptible line 15. Eighteen eggs were inoculated with 0.05 cc. of normal liver—NaCl solution (liver ratio 1:3). Seventeen controls were incubated along with the inoculated eggs for 14 days. The observations made in the four experiments are presented in table 3.

A review of the data given in table 3 shows that the mean percentage differences between the various cell types in RPL–17–injected embryos and their respective controls were so slight that an evaluation of their significance in interpreting the effects of the inoculums on the embryonic hemopoietic system is extremely hazardous.

In experiments 1, 2, and 3, about 45 percent of the injected embryos died within 5 days after inoculation, 2 percent of the eggs were infertile, and 42 percent appeared to be normal in every respect on the fourteenth day of incubation. These experiments showed that normal developmental and physiological processes were maintained in some embryos although known quantities of malignant tumor mince were injected into the yolk sac. Burmester and Prickett (*2*) maintain that strain 17 tumor mince is similar to the agent of lymphomatosis in several respects. The original inoculum used in these experiments was prepared from lymphomatotic tissues of a chicken that developed lymphomatosis on the laboratory premises. Moreover, the visceral lesions developing after inoculation of strain 17 were grossly and microscopically similar to those found in some cases of naturally occurring visceral lymphomatosis. In view of these facts it seems logical to conclude that under normal conditions the yolk sac may be infected with a tumor-producing agent without any material alteration of the developmental processes of the embryo.

It is evident from experiment 4, table 3, that grossly normal liver inoculum significantly increased the mean percentages of primitive erythroblasts, primitive erythrocytes, hemocytoblasts, thrombocytes, and acidophilic myelocytes and decreased the mean percentage of definitive erythrocytes. Sixty-seven percent of the embryos inoculated with 0.05 cc. of normal liver mince died within 5 days after inoculation. Blount (*1*) found that pullets after inoculation with liver extract likewise showed significant blood changes (hemocytoblastosis); i. e., hemocytoblasts as well as immature cells of all series were significantly increased. Chilling of young chicks and bacteria also produced analogous hemopoietic variations. Jungherr (*13*), after summarizing the literature on blood changes in the avian leukosis complex, concluded that in some forms of lymphomatosis (ocular and neural) no significant alterations take place in the blood picture, while in other forms of lymphomatosis (visceral) specific blood changes, either qualitative or quantitative in nature, are of frequent occurrence. Qualitative variations are either temporary or terminal, and less constant than those in erythroblastosis or granuloblastosis. On the basis of the observations given in the foregoing review, it seems fairly safe to conclude that hemocytoblastosis as the only criterion for diagnosis of lymphomatosis in adult chickens should be used with considerable reserve. Likewise, the use of hemocytoblastosis as a diagnostic criterion for ascertaining whether or not lymphomatosis is

egg-borne seems to be of limited value, since RPL–17 tumor mince produced no detectable blood changes in the peripheral circulation of surviving embryos. Nevertheless, liver mince prepared from grossly negative tissues did produce blood changes in the embryo which in a descriptive sense met the requirements for hemocytoblastosis.

DISCUSSION

In the preceding pages it has been shown that variations in number of hemocytoblasts and other cell types in the peripheral circulation of inbred resistant and susceptible embryos are fairly common. Obviously, there may be several interpretations of such expressions of differences in the physiological condition of the hemopoietic foci of the embryo; for example, (1) the increased numbers of hemocytoblasts in the peripheral circulation of embryos may indicate the reactivity of the hemopoietic tissues to the agent of lymphomatosis; (2) resistance to lymphomatosis in inbred chickens may be expressed in the embryo by increased numbers of hemocytoblasts in the embryonal peripheral circulation; and (3) a close correlation may exist between retardation in embryonic development and increased numbers of hemocytoblasts in the embryonic peripheral circulation.

If lymphomatosis is an egg-borne disease, transmitted from dam to offspring by means of a filterable agent or tumor cell, embryonic tissues other than peripheral blood would be expected to show manifestations of the disease. Embryos exhibiting increased numbers of hemocytoblasts were of normal size and there were no gross or microscopic evidences of infiltration or proliferation of lymphocytes in the liver or of abnormal eye conditions comparable to those found in infected adult birds. Furthermore, there was no evidence of embryonic fowl paralysis, since movement of appendages and other body parts of embryos seemed to be normal in every respect. These observations suggest that if lymphomatosis is egg-borne, there are no gross pathological manifestations of the disease in the embryo.

Assuming that genetic resistance to lymphomatosis is expressed in the embryo by increased numbers of hemocytoblasts in the embroyonal peripheral circulation, it is to be expected that some embryos in the susceptible lines will show increased numbers of hemocytoblasts, since these lines are resistant enough to supply breeding stock and sufficient numbers of chickens for experimentation. Evidence for such a differential resistance to lymphomatosis was presented in the discussion of variations in differential counts of the peripheral blood of embryos incubated for 7 and 14 days, from lines 7, 10, 11, and 13. These differences may be due to genetic heterozygosity of resistant and susceptible lines of chickens. At the present time, the various lines of chickens used for experimentation at this laboratory are not genetically stabilized either for resistance or susceptibility. Resistance to lymphomatosis in resistant lines 10 and 13 and susceptible lines 7 and 11 is expressed statistically. If and when resistance to lymphomatosis becomes more firmly established on a genetic basis, it may be possible to correlate more completely increased numbers of hemocytoblasts in the peripheral circulation with resistance to lymphomatosis.

The third explanation suggested above for increased numbers of hemocytoblasts in the peripheral circulation of embryos takes into

consideration the possible effects of retardation in embryonal development. It has been rather well established in this laboratory that embryos from certain lines of chickens show a significant developmental lag, i. e., the actual age of embryos when estimated by somite count is frequently found to be from 3 to 20 hours less than the chronological age. This discrepancy between somite age and chronological age is well illustrated by a comparison of line 7 susceptible embryos with line 10 resistant embryos incubated for 161–167 hours (table 2). The mean percentage of definitive erythrocytes in the susceptible embryos was 40.46, while the mean percentage in the resistant embryos was 65.85. The fact that the mean percentage of definitive erythroblasts with medium hemoglobin was significantly higher in susceptible embryos (18.31) than in resistant embryos (1.30) definitely establishes this developmental lag, since an analysis of the data in table 1 shows that an increase in number of definitive erythrocytes is followed by a corresponding decrease in definitive erythroblasts. The data given in the preceding pages show also that 5 of 20 embryos in line 7 (161–167 hours) had only 10 hemocytoblasts. Thus, a correlation of embryonal developmental lag with number of hemocytoblasts is untenable.

SUMMARY

Two generations of blood cells, the primitive and definitive, were found in the peripheral circulation of chicken embryos.

Primitive erythroblasts and hemocytoblasts were predominant cell types of the peripheral circulation until about the seventh day of incubation. Other cells were occasionally found, e. g., histiocytes, basophilic and acidophilic myelocytes, giant cells, thrombocytes, polymorphonuclear neutrophilic leucocytes, and degenerating cells.

On about the seventh day of incubation, significant numbers of cells of the definitive generation appeared in the peripheral circulation. The most immature erythroblast of the definitive series contained less hemoglobin and was smaller than the most immature erythroblast of the primitive series.

Giant cells were occasionally found in supravital preparations of the peripheral blood of embryos of various ages, and these cells frequently gave rise to one or more granulocytes by the production of cellular blebs. Such blebs ultimately pulled away from the surface and became free. Giant cells under other conditions fragmented to form thrombocytelike cells.

Hemopoietic tissue in the peripheral blood of embryos approached stability on about the ninth day of incubation. The mean percentage of primitive erythroblasts at about this time decreased to 5.14 while the mean percentage of definitive erythrocytes showed a corresponding increase to 90.22.

The frequency of occurrence and also the total number of hemocytoblasts in the peripheral circulation of embryos from resistant lines 13 and 10 were higher than in that of embryos from susceptible lines 7 and 11. The evidence suggests, but does not demonstrate, that increased numbers of hemocytoblasts in the peripheral circulation may be an important factor in resistance to lymphomatosis. Other resistant factors were probably operative, since embryos receiving RPL–

17 tumor mince by yolk-sac injection showed a peripheral blood picture which was essentially the same as that of uninjected control embryos.

The peripheral blood of resistant embryos with higher than normal numbers of hemocytoblasts differed markedly from that of adult chickens with lymphomatosis. In the former, all blood cells other than hemocytoblasts were essentially normal morphologically as well as numerically; in the latter, the peripheral blood of some infected adult birds frequently showed increased numbers of immature cells of the red cell series while that of others showed increased numbers of myeloblasts.

The mean percentage of definitive erythrocytes in embryos of 161–167 hours' incubation was highest in resistant line 13 (73.05), next highest in resistant line 10 (65.85), lower in susceptible line 11 (60.37), and lowest in susceptible line 7 (40.46).

Normal liver injected into the yolk sac prior to incubation significantly increased the mean percentages of primitive erythroblasts, primitive erythrocytes, hemocytoblasts, thrombocytes, and acidophilic myelocytes and decreased the mean percentage of definitive erythrocytes. Sixty-seven percent of the embryos receiving grossly normal liver mince died within 5 days after inoculation.

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